Spring 5-11-2012

Molecular and Functional Characterization of Tick Salivary VTI Protein Family

Ashley Villarreal
University of Southern Mississippi

The University of Southern Mississippi

Molecular and functional characterization of Tick salivary VTI protein family

by

Ashley Villarreal

A Thesis

Submitted to the Honors College
Of the University of Southern Mississippi
In Partial Fulfillment
Of the Requirements for the Degree of
Bachelor of Science
In the Department of Biological Sciences

March 2012

Approved by:

Shahid Karim

Professor of Biological Sciences

Glen Shearer, Chair

Department of Biological Sciences

David R. Davies, Dean

Honors College

Abstract

Tick salivary glands serves as the organ of osmoregulation in ticks and are critical to the biological success of ticks both during extended period off the host and also during the feeding periods on the host. The salivary glands are the sites of pathogen development and saliva is the route of transmission. The importance of multifunctional salivary glands to tick survival and vector competency makes the glands a potential target for intervention. The complex process by which tick salivary gland proteins exit the glands and enter the host has been extensively studied, in what is called the SNARE hypothesis, soluble N-ethylmaleimide-sensitive factor attachment proteins (SNAPs) and their receptor proteins (SNAREs) interact to move vesicles rich in tick salivary gland proteins out of the salivary gland cells. In this study, we tested the hypothesis that VTI family of SNARE proteins, VTI1A and VTI1B, are required for the salivary vesicle transport and success of tick to gain an uninterrupted access to blood-meal for several days. Consistent with previous studies, AmVti1A and AmVti1B possess 28% amino acid identity, but much higher amino acid identity to other homologs. These two proteins appear to play different roles in vesicular trafficking and exhibit distinct localization. Transcriptional gene expression using RT-qPCR revealed elevated expression of Vti1A and Vti1B during the early phase of feeding. Knockdown analysis was performed by injecting adult female *A. maculatum* ticks with dsRNA for AmVti1A, AmVti1B or both genes to further elucidate their function. Whereas Vti1A knockdown ticks did not seem to produce a significant phenotype, Vti1B knockdown ticks produced significantly lower engorgement weights than those of Vti1A knockdown ticks and those treated with a mock control. Previous research suggests that Vti1B, or other identified SNARE partners, may be able to partially compensate for the loss of Vti1A. Our results demonstrate an important role of VTI protein family in uninterrupted prolonged tick feeding on the host. Understanding the basic mechanisms of VTI protein family in salivary glands may lead to better ways to prevent tick and tick-borne diseases.

Table of Contents

Introduction

Ticks and Tick-borne Diseases

All tick species belong to class Arachnida of phylum Arthropoda. There are currently 867 identified tick species (Bowman et al., 2004). Characteristic of most arachnids, the tick body plan consists of a cephalothorax and an abdomen, both of which are easily distinguishable when examining the tick up close (Sonenshine, 1993). They also have four pairs of legs which develop in the nymphal stage, the second of the three tick stages, and they more fully develop in the adult stage, which is the final stage of tick development (Sonenshine, 1993). In general, ticks can be divided into two broad classifications, hard ticks and soft ticks, at the family level of the Linnaeus system. The hard ticks are classified under the family Ixodidae and soft ticks are classified under the family Argasidae (Sonenshine, 1993). Ticks are hematophagous, or feed on blood. Blood is the only nutritious meal taken by ticks. They are more specifically pool feeders in that the blood they obtain is drawn in by sucking it in from around a wound the tick inflicts (Bowman & Sauer, 2004). Of importance to this study are two ticks found in the *Amblyomma* genus: *Amblyomma maculatum*, the Gulf-Coast tick and *Amblyomma americanum*, the lone-star tick. The *Amblyomma* genus is currently comprised of 129 identified species of ticks which all exhibit a 3-host life cycle (Bowman et al., 2004).

Amblyomma maculatum (The Gulf Coast Tick)

A. maculatum, also known as the Gulf Coast tick, was originally primarily found along the Gulf Coast of the United States (Bishopp & Hixson, 1936). The range of the tick has, however, expanded most likely due to transportation and movement of infested livestock (Goddard & Norment, 1983) and migratory birds like cattle Egrets (USDA APHIS). Outside of the mainland USA, *A. maculatum* is found on the coastlines of other

countries bordering the Gulf of Mexico such as Mexico, Guatemala, and Costa Rica. (Estrada-Pena et al., 2005). Collections of *A. maculatum* on the Mississippi Gulf Coast show that this species favors sunny locations as opposed to shade or lots of moisture (Goddard & Stokes, 2008). This species feeds on different hosts at different stages of the life cycle; adults are common on large domestic animals such as cattle, sheep, horses, mules, deer and feral swine, whereas, larvae and nymphs generally infest birds and small mammals (Teel et al., 2010; Cooley & Kohls, 1944; Strickland et al., 1976; Greiner et al., 1984; Bishopp & Trembley, 1945).

Figure 1. Female *Amblyomma maculatum* tick

The GCT blood-feeding causes extensive inflammation, edema, abscesses, pre-disposition to myiasis, anemia and secondary infections in host animals (Bishopp & Trembley, 1945; Williams et al., 1978; Sonenshine, 1993; Hooker et al., 1912). Heavy infestation of the adult Gulf Coast tick inside the ear of animals produces inflammation, swelling, production of yellowish exudates and edema making the animal vulnerable for secondary infection. Especially in cattle, this may also lead to a condition known as "gotch ear" wherein the ears become curled and deformed due to the destruction of ear muscles and cartilage (Strickland et al., 1976; Hooker et al., 1912; Robinson, 1926; Drummond & Whetstone, 1970). Another detrimental effect that is possible from blood feeding on the host is the inoculation of the host with a toxin produced from the salivary glands. This could lead to a neuromuscular condition commonly referred to as tick paralysis

(Paffenbarger, 1951; Espinoza-Gomez et al., 2011). In severe cases, may lead to muscular and respiratory failure and ultimately death (Cupp, 1991).

The potential for *A. maculatum* as a vector for disease is also not a new matter of discussion, and it continues to be one of importance. In 1937, Dr. Ralph R. Parker first discovered the presence of *Rickettsia parkeri* in *A. maculatum* (Parker et al., 1939). Originally thought to be non-pathogenic to humans, the first documented human infection did not occur until 2004 (Paddock et al., 2004). The *Rickettsia parkeri* agent is now known to cause a type of spotted fever similar, but not identical, to Rocky Mountain Spotted Fever (Paddock, 2004). The activity of *A. maculatum* as a vector for *R. parkeri* seems to be increasing, with relatively high (10-28%) infection prevalence reported in several areas (Sumner et al., 2007; Paddock et al., 2010, Wright et al., 2011). *A.maculatum* has also been identified as a vector for *Ehrlicia ruminatum* which is the pathogen for Heartwater (Walker & Olwage, 1987). Due to the high prevalence of heartwater in the Caribbean and the United States' close proximity, the transmission of this pathogen is a serious threat that has potential to cause severe damage to livestock and wildlife in the United States (Burridge, 1997). The spread of *E. ruminantium* to North America is especially important given that migrating birds can bring ticks harboring infectious foreign agents. The threat for the importation of *E. ruminantium* to the mainland USA by bird migration is a continued public health concern because *A. maculatum* has already been identified as a competent tick vector (Mahan et al., 2000).

***Amblyomma americanum* (The Lone Star Tick)**

Amblyomma americanum was the first tick ever identified in the United States in 1754 (Childs and Paddock 2003). This tick species is more commonly referred to as the

Lone Star tick and has a more widespread inland distribution than the Gulf Coast tick (Keirans & Lacombe, 1998). More specifically the distributuion of *A. americanum* ranges from Texas up to Oklahoma and then East, extending all the way north into Maine (Hair & Bowman). Whereas *A. maculatum* seems to favor sunny areas, the most preferred habitat of the Lone Star tick seems to be more wooded areas with significant amounts of underbrush (Hair & Howell, 1970).

Figure 2. Female *Amblyomma americanum* tick

Due to their form of nutrition, ticks are inherently parasitic; however, ticks can potentially cause even more damage, economically and medically, as they serve as vectors for disease. Multiple tick borne diseases have already been recorded (Goddard & Stokes, 2008). *Amblyomma americanum* was originally not thought to pose a very large threat as a vector, a thought which remained consistent until quite recently. In the 1990's, the Lone Star tick gained more attention as it was implicated as a vector for *Ehrlichia* species (Hair & Howell, 1970). *Amblyomma americanum* is now strongly linked to the spread of *Ehrlichia chaffeensis* which is a disease agent which causes ehrlichiosis or even death in some cases (Goddard & Stokes, 2008). This species has also been implicated as a vector for screw worm and tularaemia (Bowman et al., 2004).

In the past decade, the Lone Star tick has been evaluated as a potential vector for a more well known disease, Lyme disease. This disease is only known to be caused by the spirochete *Boorelia burgdorferi*, which has previously only been known to be transmitted

by ticks from the *Ixodes* genus (Wang et al., 1999). Most incidences of Lyme disease occur in the north and northeastern regions of the United States, where *A. americanum* is not distributed quite as densely (CDC 1999). However, the identification of patients in more southern states that exhibited the same type of illness resulting from Lyme disease accompanied by a rash after a documented bite from *A. americanum* led to the suspicion that *A. americanum* may also be a vector for Lyme disease (Campbell et al., 1995, Kirkland et al., 1997) Further analyses of these cases and PCR amplification of the spirochete, led to the identification of a new *Boorelia* species named *Boorelia lonestari* named so for the supposed transmission by the Lone Star tick (Barbour et al., 1996, Armstrong et al., 1996). More studies are being done to better characterize this spirochete and its connection to Lyme disease, the most prevalent vector borne disease.

If any progress is to be made in the efforts to control *A. maculatum* and *A. americanum* as potential vectors, studying the ticks' feeding mechanisms may provide the best insight to their success. As bloodfeeders, ticks are inherently ecological parasites; however, when the tick is serving as a vector, the damage it can cause increases dramatically. In most mammals, there are defense mechanisms set up to prevent blood loss when the skin is punctured. This "plug in the hole" type of effect that occurs because of a chain reaction begun by the binding of tissue factor to activated factor VII is called the "hemostatic response" (Hovius et al., 2008). It is the tick's challenge to find a way around these mechanisms in order to obtain their food. Judging by the efficiency of the tick in feeding and spreading disease agents, there are most likely some internal biological factors at work.

Salivary Glands

Although these tick species are well-known vectors in the spread of disease, how they take up, harbor and distribute the pathogen(s) is still poorly understood in many cases. Part of the tick that seems to play an important role and feeding and thus pathogen transmission are the salivary glands. Although in other organisms, such as humans, salivary glands play a smaller role in the aiding of lubrication and digestion, the salivary glands are much more vital to ticks (Sauer & Hair, 1986). In general, tick salivary glands aid in rehydration of the tick between blood meals and also the release of important components that help the tick attach and feed successfully.

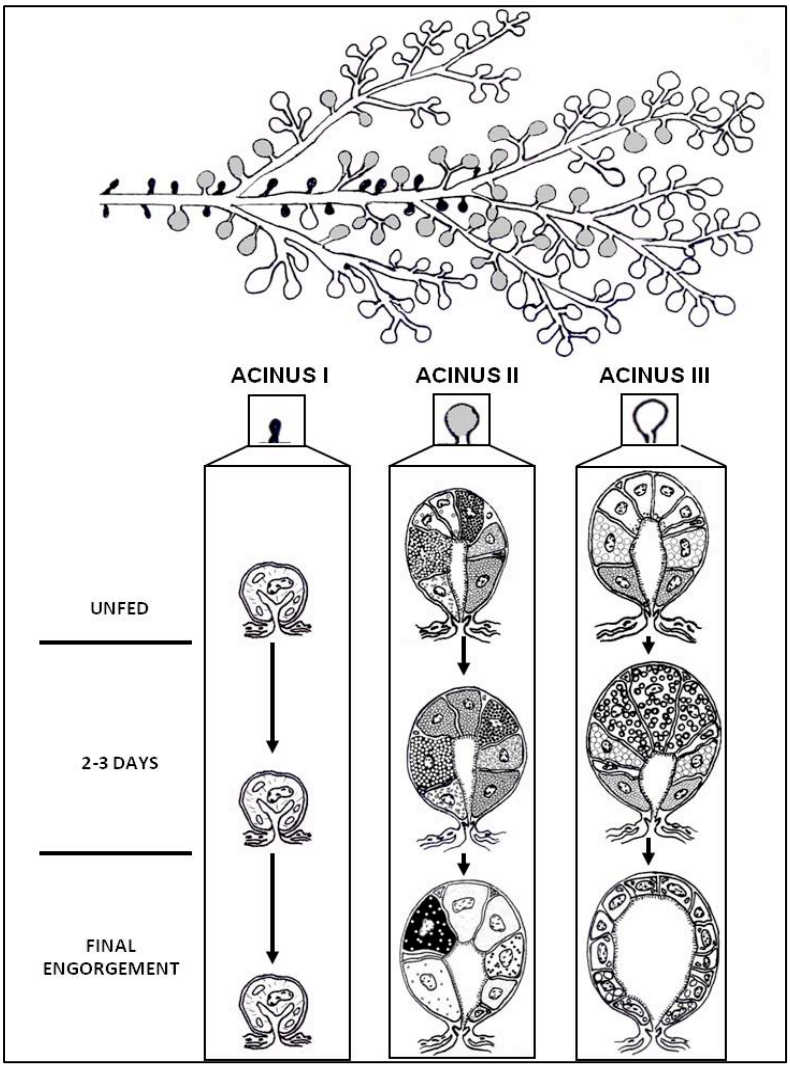

Figure 3. Depiction of a tick salivary gland and the development of its acini throughout the blood meal

There are three types of acini which comprise the salivary glands that seem to differ in morphology and function. Acini I, the smallest of the three acini, are most

directly involved in osmoregulation (McMullen et al. 1976; Rudolph & Knulle, 1974). Type II acini are composed of six different cell types which have a variety of granules that may function in fluid and electrolyte transport (Sauer & Hair, 1986). The most studied of the three acini, type III, exhibit significant structural changes during feeding and are composed of three glandular cell types (Tatchell, 1967). Acini III have been implicated to have a large number of granules and may be responsible with concentrating the blood meal (Sauer & Hair, 1986). During the lengthy blood meal of Ixodid ticks, the cells of the salivary glands grow and differentiate as the amount of proteins and other cell mass increase almost 25 fold. Upon repletion, the cells quickly return to their unfed state (Sauer et al., 1995).

Salivary Secretions

As stated before, the tick saliva carries proteins vital to the tick attachment and feeding on the host; therefore, the secretion of this saliva is crucial to tick survival. Within the saliva, many types of proteins are found that aid in the success of the tick such as anticoagulants, anti-inflammatory proteins, immunosuppressants and prostaglandins (Sauer et al. 1995; Lees & Bowman, 1997). The releasing of saliva has been determined to be controlled by the tick's central nervous system (Sauer et al. 1995). The key neurotransmitter is dopamine. In response to the signaling of dopamine, there is an increase in the levels of cAMP, which is responsible for stimulating fluid secretion, and Ca 2+, which increases the release of arachidonic acid. This arachidonic acid then can be converted into prostaglandins one of which is PGE2. In addition to other functions, PGE2 mobilizes intracellular Ca2+ which promotes final docking and fusion events of vesicles and thus the release of salivary proteins (Sauer et al., 2000).

Exocytosis

In order to better understand how the contents of the salivary glands are released from the cell, the process of exocytosis must be more closely examined. Although the steps of salivary secretion have been detailed, the process involves even more steps in order to secrete the vesicle from the cell. Within the process of exocytosis from transport from ER to golgi and eventually to the plasma membrane, if the product is to be secreted, there exist multiple vesicular intermediates (Bennet & Scheller, 1993). This idea of vesicular mediation suggests that each step of docking and fusion between the different components of the cell also requires specific machinery. Also this process becomes more complex as final docking and fusion events can be dependent on other molecules (Bennet & Scheller, 1993). This is the case for tick salivary secretions, as before mentioned final docking and fusion events are Ca2+ dependent.

SNAREs

Believed to be driving this process of vesicular intermediate docking and fusion and finally exocytosis of salivary proteins from the cell are the soluble NSF-attachment protein (SNAP) receptors, better known as SNAREs (Sollner et al., 1993). So far, 24 members in *Saccharomyces cerevisiae*, 21 in *Aspergillus oryzae*, 36 in humans, 68 in *Arabidopsis thaliana*, and 20 in *Drosophila melanogaster* have been identified. The sialome data reveal a full-set of ~40 SNARE protein members in *A.*

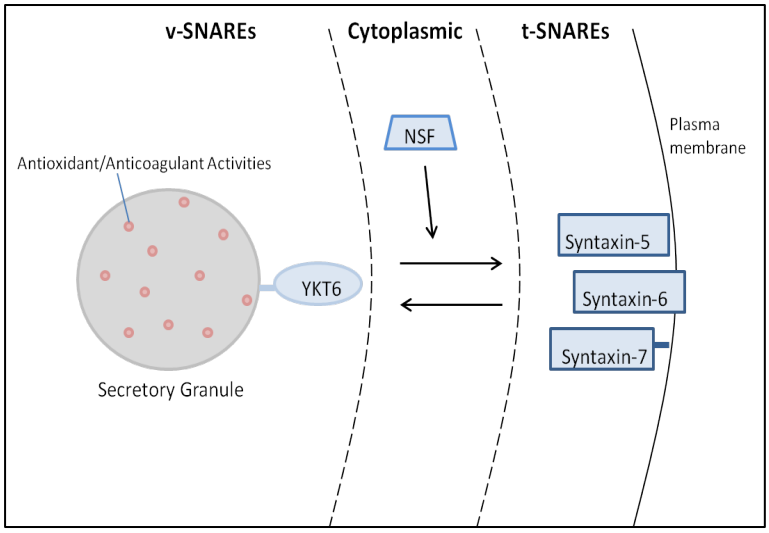

Figure 4. A working model of SNARE protein interactions

maculatum. We have already demonstrated the presence of SNARE proteins in *Dermacentor variabilis*, *Ixodes scapularis*, *Amblyomma americanum,* and *Amblyomma maculatum* salivary glands (Karim et al., 2002; 2011; Karim and Adamson 2012). The SNAREs are integral membrane proteins that exhibit similar structure with slight variations and exceptions noted in few (Jahn & Scheller, 2006). SNAREs can be generally, but not exclusively, separated into the categories of v-SNAREs and t-SNAREs, the former associated with the vesicle membrane and the latter with the target membrane (Jahn & Scheller, 2006). The formation of relatively stable complexes is made by four SNARE proteins. Through sequence analysis, it has been determined that one of the four is classified as an R-SNARE which provides an arginine residue to the functional component, and the other three are classified as Q-SNAREs which provide glutamine to the structure (Fasshauer et al., 1998). The SNARE complex that is formed works as a machine to aid in fusion of a vesicle with the target membrane (Hong, 2005). SNAREs can be involved in early steps of the secretory pathway such as between the ER and the golgi or in the exocytosis of vesicular contents.

Vti1A and Vti1B

Whereas the structure of SNARE complexes has been determined, there is still much to be studied in terms of the individual SNARE proteins and their specific roles. Of interest to this particular study are the SNARE proteins from the VTI family, Vti1A and Vti1B. VTI stands for vesicle transport through interaction with t-SNAREs. In mice, Vti1A and Vti1B exhibit only approximately 30% amino acid identity and also the same amount of homology to yeast vti 1p (Advani et al., 1998). Various studies suggest that the two do have specific roles and distinct locations (Kreykenbohm et al., 2002). Through

immunofluorescence, mouse Vti1A is shown to reside primarily in the Golgi and the TGN, and Vti1B is more highly associated with early and late endosomes (Kreykenbohm et al., 2002). Different SNARE partners have also been identified for both. Vti1a participates in a complex with syntaxin-16, syntaxin-6, and VAMP-4, and Vti1B forms a complex with syntaxin-7, syntaxin-8, and endobrevin/ VAMP-8 (Kreykenbohm et al., 2002, Antonin et. al 2000-a). In yeast, although encoded by a single gene, it can form SNARE complexes with five syntaxins (Abeliovich et al., 1999; Fischer et al., 1999).

In addition to distinct localization, particular studies also support the idea that Vti1A and Vti1B play different roles in vesicular trafficking. In *A. maculatum*, Vti1A is composed of 624 bp (partial transcript) and Vti1B is composed of 684 bp, exhibiting a head and tail region that Vti1A lacks (Karim lab). Vti1A has been specifically implicated in a regulatory step in GLUT4 and Acrp30 trafficking in 3T3-L1 adipocytes (Bose et al., 2005). Vti1A also has a variant identified as Vti1A-β, which differs in sequence by a 7 amino acid insertion at position 114 and plays a specific role in synaptic vesicles (Antonin et al., 2000-b). Vti1B, on the other hand, has been implicated to cause the loss of a SNARE complex partner and slight phenotypic variation in Vti1B -/- knockout mice (Atlashkin et al., 2003). Also in mice, a deficiency of Vti1B led to an initial reduction in the exocytosis of lytic granules from cytotoxic T lymphocytes (Dressel et al., 2010). Other studies suggest that, despite their specific locales and roles, in the case of a Vti1A-/- or Vti1B -/-, the two can substitute for one another to a functional degree (Surpin et al., 2003, Kunwar et al., 2010). However, Vti1A-/- and Vti1B -/-, or DKO organisms, showed significant phenotypic change and even death in both plants and mice, thus

further suggesting the importance of Vti1A and Vti1B in vesicular transport (Surpin et al., 2003, Kunwar et al., 2010).

Experimental Design

For this particular study, it may be important to note that the author was an undergraduate student conducting research in the laboratory of Dr. Shahid Karim. While the original purpose of this research was to better isolate the roles of Vti's in A. maculatum ticks, some previous work from the laboratory on Vti1B in A.americanum has been included and expanded upon for a comparative and more comprehensive research project. In addition, it should be noted that while this was a project of large scale which the author put countless hours of work into producing others in the Karim lab should be acknowledged for their assistance.

Ticks

Amblyomma maculatum and *Amblyomma americanum* ticks were raised and obtained from Oklahoma State University's tick rearing facility, according to the methods of Patrick and Hair (1975). Adult ticks were fed on sheep specifically for this study and all studies with animals were performed in accordance with protocols #10042001 and #08110401 approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Southern Mississippi. All unfed ticks were maintained in a 27-28 °C environment with 90% relative humidity under 14 h light/dark photoperiod before infestation of the hosts. Within 4 h of being removed from the host, salivary glands were dissected from the ticks (Karim et al., 2002).

Tick Salivary Glands

A. maculatum salivary glands were dissected in ice- cold 100 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) buffer containing 20 mM ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), pH 6.8. After removal, the glands were gently washed in the same ice-cold buffer. The tissues were then

immediately stored in RNAlater (Ambion, Austin, TX, USA) prior to isolating total RNA. Tissues were either used immediately or after dissection, or stored at -80°C in 0.5 mM EGTA, 1x Complete Mini Protease inhibitor cocktail (Roche, Indianapolis, IN, USA), and 40% glycerol for Western blotting. All other manipulations were carried out at 4 °C.

Bioinformatics

The full length *A. maculatum* Vti1A and *A. maculatum* Vti1B sequences were obtained from pyrosequencing an *A. maculatum* salivary gland cDNA library. Sequences for comparative analysis of *A. maculatum* Vti1A were obtained from the NCBI database with the following accession numbers: *Ixodes scapularis* (GenBank ID: XP_002406341.1), *Homo sapiens* (GenBank ID: NP_660207.2), *Canis lupis familiaris* (GenBank ID: XP_003433671.1), *Taeniopygia guttata* (GenBank ID: XP_002198053.1), *Rattus norvegicus* (GenBank ID: NP_075589.1), *Mus musculus* (GenBank ID: NP_058558.1), *Pediculus humanus corporis* (GenBank ID: XP_002425289.1), *Solenopsis invicta* (GenBank ID: EFZ09227.1), *Danio rerio* (GenBank ID: NP_001030152.1), *Culex quinquefasciatus* (GenBank ID: XP_001848598.1), *Tribolium castaneum* (GenBank ID: XP_970611.1), *Ciona intestinalis* (GenBank ID: XP_002122705.1), *Caenorhabditis elegans* (GenBank ID: NP_502781.1), *Drosophila melanogaster* (GenBank ID: NP_612053.1), *Daphnia pulex* (GenBank ID: EFX61974.1), and *Arabidopsis thaliana* (GenBank ID: NP_564255.1). Sequences were aligned using ClustalX2, and Jalview 2.7 was used to graphically present the data. Phylogenetic relationships were inferred using MEGA 5. Protein secretion signals were identified using SignalP 3.0. The same process was followed for A. maculatum vti1b using the

following accession numbers: *A. americanum* (ACG76200.1), *Mus musculus* (GenBank ID: NP_058080.2), *Danio rerio* (GenBank ID: NP_957330.1), *Canis lupis familiaris* (GenBank ID: XP_003640058.1), *Pan troglodytes* (GenBank ID: XP_003310626.1), *Gallus gallus* (GenBank ID: XP_421192.1), *Taeniopygia guttata* (GenBank ID: XP_002198053.1), *Homo sapiens* (GenBank ID: BAG38161.1), *Pongo abelli* (GenBank ID:XP_002824912.1), *Equus caballus* (GenBank ID: XP_001499987.3), *Ciona intestinalis* (GenBank ID:XP_002130133.1), *Monodelphis domestica* (GenBank ID: XP_001368381.1), *Arabidopsis thaliana* (GenBank ID: Q9LVP9.1), *Culex quinquefasciatus* (GenBank ID: XP_001848843.1), and *Macaca mulatta* (GenBank ID: XP_001093266.1).

cDNA Synthesis and RT-PCR

Using tick-specific EST sequences for AM Vti1A and AM Vti1B, gene-specific primers were designed to amplify cDNA fragments from salivary glands of the gulf coast tick. Total RNA extraction from salivary glands and midguts of partially fed female gulf coast ticks was done using a RNAaqueous total RNA isolation kit (Ambion). The total RNA concentration was measured spectrophotometrically. Before use, it was aliquoted and stored at -70 °C. Reverse transcription of total RNA was done using Moloney murine leukemia virus (M-MLV) reverse transcriptase according to the Invitrogen protocol. For each gene, cDNA was PCR amplified using gene-specific primers for tick salivary gland vti1a (forward, 5'-ATGATTGGCATTCTGAAAATGGCGA-3' and reverse, 5'-TCACACATGCCTTCGAACTGTA AAA-3') and tick salivary gland vti1b (forward, 5'-ATGTCCTCGGAAAAGTTTGAGGA-3'and reverse, 5'-TCACTTCATGATAAACTTC

CAGTAGATAA-3'). PCR was executed with a program of 94 °C for 1 min, 29 cycles of 94 °C for 1 min, 49 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 8 min.

Transcriptional Expression with qRT-PCR

The cDNA was diluted to approximately 25ng/µl for qRT-PCR gene expression analysis. First strand cDNA was used to measure mRNA levels through qRT-PCR. For quantification with BIORAD CFX96 Real Time System, the Maxima SYBR Green qPCR Master Mix (Fermentas) was used according to manufacturers recommendations; approximately 50 ng of cDNA and gene specific primers (150 nM) were used for each reaction mixture. The C1000 Thermal Cycler was used to control the thermal treatment which was 10 minutes at 95 °C, followed by 35 cycles of 15 seconds at 95 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C.

Cloning and Sequencing

After PCR amplification, gel-purified fragments were cloned into the pCR-II vector using a TOPO T/A cloning Kit (Invitrogen). Recombinant plasmids were then transformed into TOP 10F' One Shot Competent Cells and cultured on agar plates containing 50 µg/ml ampicillin, 0.2 mM IPTG, and 40 µg /ml X-gal for selection and amplification. White colonies (8 colonies) were picked from these plates and grown in Luria Broth (LB) containing 50 µg/ml ampicillin. Plasmid DNA was purified with Qiaprep spin Miniprep plasmid kit (Qiagen). Cloned recombinant plasmid DNA (250 ng) was tested for the presence of an insert by comparing its migration to the vector plasmid alone on 2% agarose gel electrophoresis. Positive clones were then digested with HindIII and NheI. To verify the insert, the product was analyzed again by agarose gel electrophoresis and visualized on a 2% agarose gel. Those PCR products deemed to have

the insert were sequenced using Beckman-Coulter CEQ 8000 Genetic Analysis System in the Genomics and Sequencing Center at URI. The resulting sequences were used to search the DNA and protein databases (www.ncbi.nih.gov/blast).

In vivo Gene Silencing

PCR products of Vti1A and Vti1B were joined to the Block-iT T7 TOPO linker. This TOPO linking reaction was used in two PCRs with gene-specific and T7 PCR primers to produce sense and antisense linear DNA templates. These sense and antisense DNA templates were then used to generate sense and antisense transcripts of using the BLOCK-iT RNA TOPO transcription kit (Invitrogen). The sense and antisense ssRNAs were purified and then mixed together, incubated at 65 °C for 5 min, and later left to anneal at room temperature for 4 h to make dsVti1A and dsVti1B respectively. To make the double knockout, appropriate amounts of dsVti1A and dsVti1B were mixed. For each type of dsRNA, 50 unfed female ticks were injected with 500 ng dsRNA using a 33-gauge needle. After injection, ticks were kept overnight in a 37 °C water bath to monitor tick survival after injection. All surviving PBS buffer and dsRNA injected ticks were infested on separate cells on the sheep for feeding.

Post Feeding Analysis

Ticks were allowed to blood feed and then pulled off periodically or allowed to feed until repletion. Feeding success was determined by survival and engorged weight. Ticks that had a mass of over a few hundred milligrams were cleaned with distilled water and placed in an incubator for observation of egg laying capabilities. An ANOVA statistical analysis was used through the SigmaPlot software in order to determine significantly different tick masses among groups as compared to the control group.

Transcriptional expression levels were measure by the method previously described in a prior section using qRT-PCR.

Confocal Imaging

For immunolocalization, dissected salivary glands from partially fed females were stored in 4% paraformaldehyde at 4°C. Dissected glands were rinsed in PBS for 30 minutes then soaked in 4% formaldehyde in PBS for 30 minutes at room temperature then washed in PBS. Glands were permeabilized in 0.5% tritonX-100 for 30 minutes at room temperature and washed in PBS. Next, glands were incubated in 3% BSA for 1 hour at room temperature and excess BSA was washed away with PBS. Glands were soaked in the primary antibody (1:100) in 3% BSA and incubated at 4°C for overnight. Glands were washed in PBS. Salivary glands were incubated in Alexa-546 anti-mouse secondary antibody (1:100) in 3% BSA for 1 hour at room temperature in the dark. Excess antibody was rinsed away with three PBS washes. Glands were incubated in DAPI for 5 minutes and thoroughly washed with PBS at room temperature. Glands were mounted on glass slides and viewed under a Leica TCS 4D confocal microscope.

Results

Homology in VTI family

Multiple sequence alignment of AM Vti1A showed high conservation with other model organisms and 79% amino acid identity to *Ixodes scapularis*, the deer tick (Fig 1A). Multiple sequence alignment of AM Vti1B showed similar conservation, and 94% amino acid identity with our other species of interest the Lone Star tick, *Amblyomma americanum* (Fig 1B). Upon comparative sequence alignment of AMVti1A and Am Vti1B, only 28% sequence identity was exhibited. As stated previously, this low degree of homology within the VTI family is consistent with what has been found in other species (Advani et. al 1998).

Transcriptional gene expression and localization of Vti1A and Vti1B

In order to better assess the roles of AM Vti1A and AM and AA Vti1B during prolonged blood-feeding, qRT-PCR was used to check the expression of Vti1A and Vti1B in unfed and partially fed salivary glands. AMVti1A exhibited high expression in the unfed salivary glands, but showed a significant decrease in transcript level which remained fairly consistent throughout the feeding phases (Fig 6). The expression of Vti1B is also high in the unfed salivary glands, but the transcriptional level remains relatively high throughout the feeding phases reaching a peak at 36h which is during the early feeding phase (Fig 7). However, in *A. americanum*, Vti1B reaches a peak at 5d which is the end of the slow feeding phase (Fig 8).

In vivo Gene Silencing Effects

A. Transcriptional gene expression

Hallmarks of RNAi are its specificity, simplicity and the ease by which the

method can be used to ticks in vivo. Through the technique of RNA interference, unfed ticks were injected with either ds-RNA Vti1A, Vti1B or both to create a double knockdown. After allowing the ticks to feed, qRT-PCR checking for transcriptional gene expression revealed the effectiveness of the RNAi to deplete the levels of the desired transcript and to see the effect it had on its VTI counterpart. As compared to the gene transcript levels in naïve *A. maculatum* ticks, Vti1A transcript levels were reduced to 88% (Fig 11). AM Vti1B transcript levels were more effectively reduced by 99% of the transcripts in naïve ticks (Fig 12). In the double knockdown, AM Vti1A transcripts were only reduced by 77%, but AM Vti1B transcripts were reduced significantly more by 98% (Fig 13). In the *A. americanum* ds-RNA Vti1B injected ticks, the transcript levels were reduced by 99% (Fig 14).

B. Feeding and Fecundity

Ticks were either allowed to feed to repletion or pulled off periodically throughout the feeding phases to assess and compare engorgement weights. In the *A. maculatum* sample from 6 days (Fig 15), ds-RNA Vti1B injected ticks exhibit a noticeably smaller phenotype than that of the PBS control; however, the double knock down ticks do not exhibit this phenotype and are relatively the same size as the control ticks. The engorgement masses of all ticks were obtained. The average tick mass was 83.69 mg in Vti1A knockdown ticks, 27.18 mg in Vti1B knockdown ticks, 211.72 mg in double knock down ticks and 228.46 mg in the PBS control. Only Vti1B knockdown ticks were found to be significantly different masses as compared to the PBS control through ANOVA analysis (Fig 16). In the *A. americanum* tick sample at 5 days (Fig 17), the same reduced phenotype was observed as compared to the LacZ control ticks.

Statistical analysis of individual tick masses also showed that *A. americanum* dsRNA-Vti1B injected ticks were significantly smaller than the LacZ injected control ticks (Fig 18). The average tick mass was 32.84 mg in Vti1B knockdown ticks and 83.26 mg in the Lac Z control treated ticks.

C. Immunolocalization

Antibody-antigen interactions were used to help determine localization of Vti1A and Vti1B within the salivary glands. Figures 9 and 10 show the Vti1B localization in the salivary cells of unfed *A. americanum* and *A. maculatum* ticks. Vti1B is highly expressed in both the *A. maculatum* and *A. americanum* unfed salivary glands. Figure 15 shows the localization of AM Vti1A in the control and various knockdown ticks. Vti1A is highly expressed in the PBS control salivary glands exhibiting localization to the salivary duct. The level of expression of Vti1A in the Vti1A knockdown shows the significant reduction of the Vti1A gene transcripts as was previously indicated by the qRT-PCR. Some expression of Vti1A remains in the Vti1B knockdown. In the double knockdown, Vti1A is still found at a significant level in the salivary glands possibly due to the fact that gene transcript levels were only reduced by 70%. Figure 16 shows the same types of images for AM Vti1B. For Vti1B, expression is high in the PBS control. Vti1B was effectively reduced in the Vti1B knockdown as significant protein depletion was observed using immunofluorescence detection . Vti1B was localized throughout the acini in the Vti1A knockdown sample possibly suggesting compensatory mechanisms in place. In the double knockdown, Vti1B was again effectively reduced. Localization of AA Vti1B was also determined in partially fed *A. americanum* ticks but is not as distinct as that seen in *A. maculatum* (Fig 17).

D. Effect on other SNAREs

As it has been suggested that other SNAREs can sometimes compensate in the case of a knock down of other SNAREs, the transcriptional gene expression of other identified SNAREs was observed (Fig 20). As compared to the control, Syntaxin 8 and Syntaxin 16 seem to be slightly downregulated in the case of all three knockdowns. VAMP7 seems to be slightly upregulated in the double knockdown.

Discussion

Both *A. maculatum* and *A. americanum* have been implicated as vectors for numerous diseases. The internal mechanisms of the tick to transmit these pathogens are of great interest in the search to achieve better vector control. Tick saliva contains numerous bioactive proteins as documented from biochemical analysis of secretagogue induced saliva (Sauer et al., 1995; Sauer et al., 2000, Francischetti et al., 2009; Ribeiro & Francischetti, 2003). A high degree of conservation has been found in the intracellular SNARE complex proteins associated with the mechanism of exocytotic protein secretion in vertebrate and invertebrate neuronal and non-neuronal cells. Due to their seemingly integral role in this exocytotic process, the SNAREs have become a major point of interest in membrane trafficking research (Hay & Scheller, 1997). Our studies indicate that SNARE complex proteins are most likely involved in Ca^{2+} regulated protein secretion in tick salivary glands (Karim et al., 2002, 2011). Eukaryotic cells contain several SNARE proteins involved in vesicle transport pathways. The VTI (vesicle transport through interaction with t-SNARE homolog) proteins have multiple functions. In yeast, although encoded by a single gene, it can form SNARE complexes with five syntaxins (Abeliovich et al., 1999; Fischer et al., 1999). In mouse, MmVTI1a is localized to the Golgi and involved in intra-Golgi trafficking. MmVTI1b is found on the Golgi, the TGN, and possibly the endosome (Kreykenbohm et al., 2002). VTI1A participates in a complex with Syntaxin-16, Syntaxin-6, and VAMP-4, and VTI1B forms a complex with Syntaxin-7, Syntaxin-8, and endobrevin/VAMP-8 (Kreykenbohm et al., 2002; Antonin et al. 2002).

From our bioinformatics study, Vti1A and Vti1B were found to be conserved across species possibly suggesting a conserved role in vesicular transport (Fig.1-2). As suggested by Kreykenbohm et al. 2002, Vti1A and Vti1B do seem have to have distinct localization and play different roles in the tick species as well. The results from confocal imagery of Vti1A and Vti1B (Fig.9-10, Fig.15-17) helped to display this in a visual manner in control as well as knockdown tick salivary glands showing that Vti11A is somewhat localized to the salivary duct, whereas Vti1B is expressed more ubiquitously. Also, through the use of Vti1A and Vti1B specific antibodies it was shown that in control treated tick salivary glands both VTI's are highly expressed. This technique also helped to validate effective knockdowns of Vti1B and further show how Vti1A still showed some expression in its knockdown. Future immunolocalization work coupled with more specific antibodies for proteins previously identified in vesicular transport may help to more distinctly describe specific locations for Vti1A and Vti1B in the cells of the salivary glands. Through the use of phylogenetic analysis, the VTI family of related hard ticks was found to be very closely related (Fig.4-5). Vti1B in *A. maculatum* and *A. americanum* exhibited very high similarity, thus providing greater validity to the comparisons that can be made in the resulting phenotypes of the two species.

Transcriptional gene expression of Vti1A and Vti1B may also reveal pertinent information in the comparison of Vti1A and Vti1B and their roles. In *A. maculatum*, Vti1B transcript levels were significantly higher than transcript levels for Vti1A throughout the feeding phases (Fig.6-7). This possibly suggests that Vti1B may be more important for vesicular transport and the releasing of salivary proteins in *A. maculatum*.

As for AA Vti1B, gene transcript levels showed an increase at the end of the slow feeding phase, but reduced engorged tick weights were still observed in *A. americanum*.

Reduced engorged tick weights were observed for both *A. maculatum* and *A. americanum* ds-RNA Vti1B injected ticks as compared to the control groups (Fig.18,Fig.20). However, this change was not observed in either the ds-RNA Vti1A injected ticks or the double knock down ticks. The lack of reduced engorged tick weights in the Vti1A knockdown ticks could be due to a few different factors. As stated previously, Vti1A transcript levels were generally lower than Vti1B transcript levels suggesting that Vti1A does not play as large as a role as Vti1B in the releasing of crucial salivary proteins and thus would not have a significant impact on tick feeding. Also through the use of RNA interference, Vti1A transcripts in the ds-RNA Vti1A injected ticks were only reduced to 88% as compared to the naïve ticks (Fig.11). While the inability to reduce the transcript levels by a greater percentage could have been due to human error, other studies suggest that some SNAREs are not able to be effectively reduced through RNAi (Bethani et al., 2009).

Previous research suggested that in the case a single knockout of one of the VTI's did not result in significant phenotypes due to compensation by one or the other; however, in the case of a double knockout phenotypic changes and loss of vitality were clearly observed (Surpin et al., 2003, Kunwar et al., 2010). This does not seem to hold true for these two species of *Amblyomma* ticks. Whereas the Vti1B knockdown did produce a change in feeding, the double knock down showed no change in feeding or tick engorgement weights contrary to what was expected. This could be due to the believed compensatory mechanisms of other SNAREs when both of the VTI's are knocked down.

The lack of change in the double knockdown ticks also could have been due to the inability to reduce Vti1A transcript levels by more than 77% in the salivary glands (Fig.13). This could have been due to the same reasons mentioned above for the Vti1A knock down ticks. Also as seen transcriptional gene expression of other SNAREs, VAMP7 seemed to be slightly upregulated in the double knockdown possibly suggesting some sort of compensatory mechanism. However, it is unclear if this was actually a significant upregulation that would be capable of compensating for the loss of both Vti1A and Vti1B.

Although no certain conclusions can be made at this time in regards to the Vti1A knockdown and double knockdown ticks, the reduced engorged tick weights observed in both *A. maculatum* and *A. americanum* must be acknowledged. Due to the close relation of Vti1B in the two species and similar results of feeding as compared to controls, it can be concluded that Vti1B does play an essential role in tick feeding of both *A. maculatum* and *A. americanum*. This study and continued research may help lead to a mechanism of control of *A. maculatum* and *A. americanum* as vectors for the various detrimental diseases described previously.

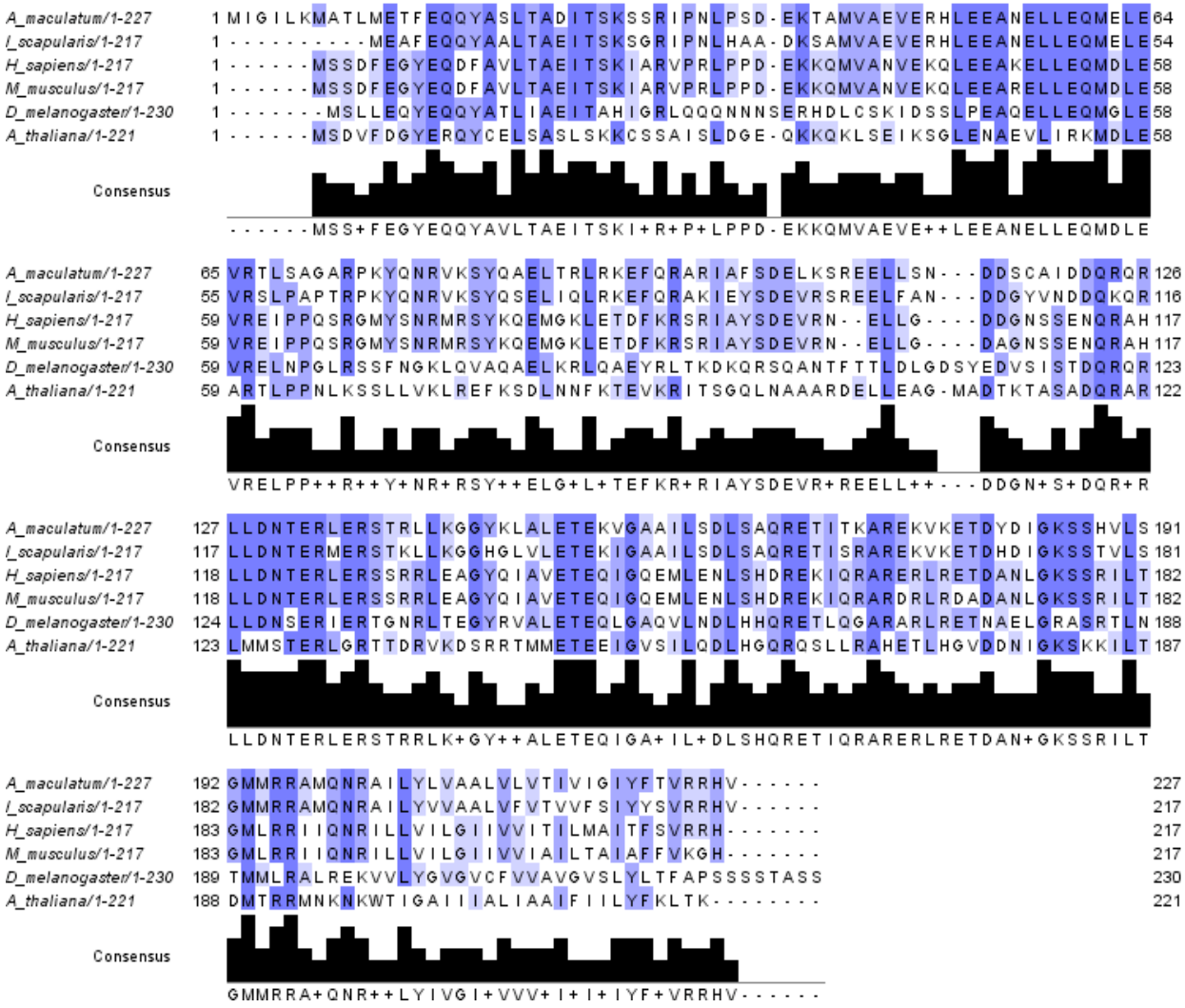

Figure 1. Multiple Sequence Alignment. The *A. maculatum* Vti1a amino acid sequence was aligned with sequences obtained from NCBI. The *A. maculatum* sequence shows 79% identity (91% similarity) to that from *I. scapularis,* 39% identity (64% similarity) to another arthropod *D. melanogaster*, but 53% identity (76% similarity) to the *H. sapiens* Vti1a sequence.

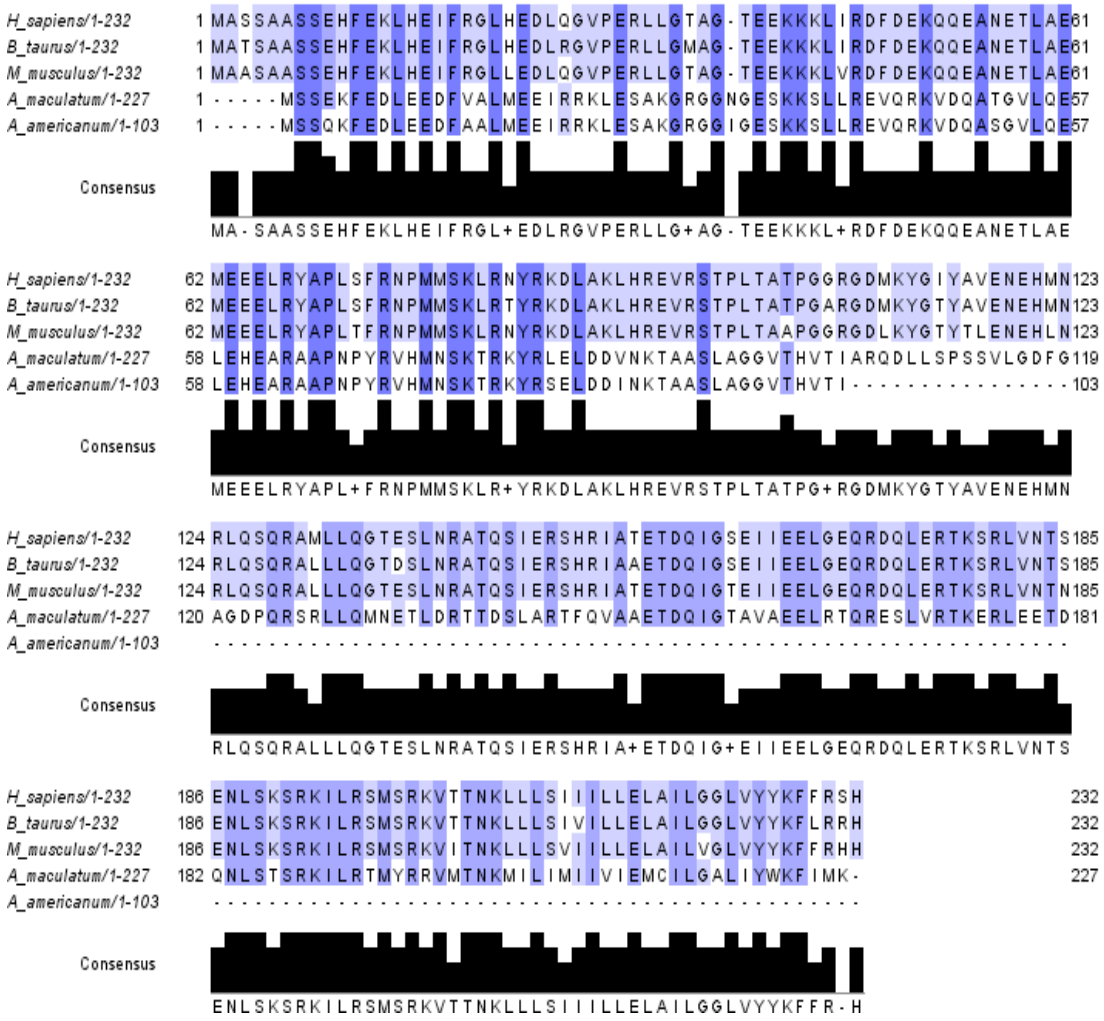

Figure 2. Multiple Sequence Alignment. The *A. maculatum* Vti1b amino acid sequence was aligned with sequences obtained from NCBI. The *A. maculatum* sequence shows 94% identity (97% similarity) to the partial sequence from *A. americanum*, and 42% identity (58% similarity) to the *H. sapiens* Vti1b sequence. No significant homology was observed between any *I. scapularis* protein and the *A. maculatum* sequence.

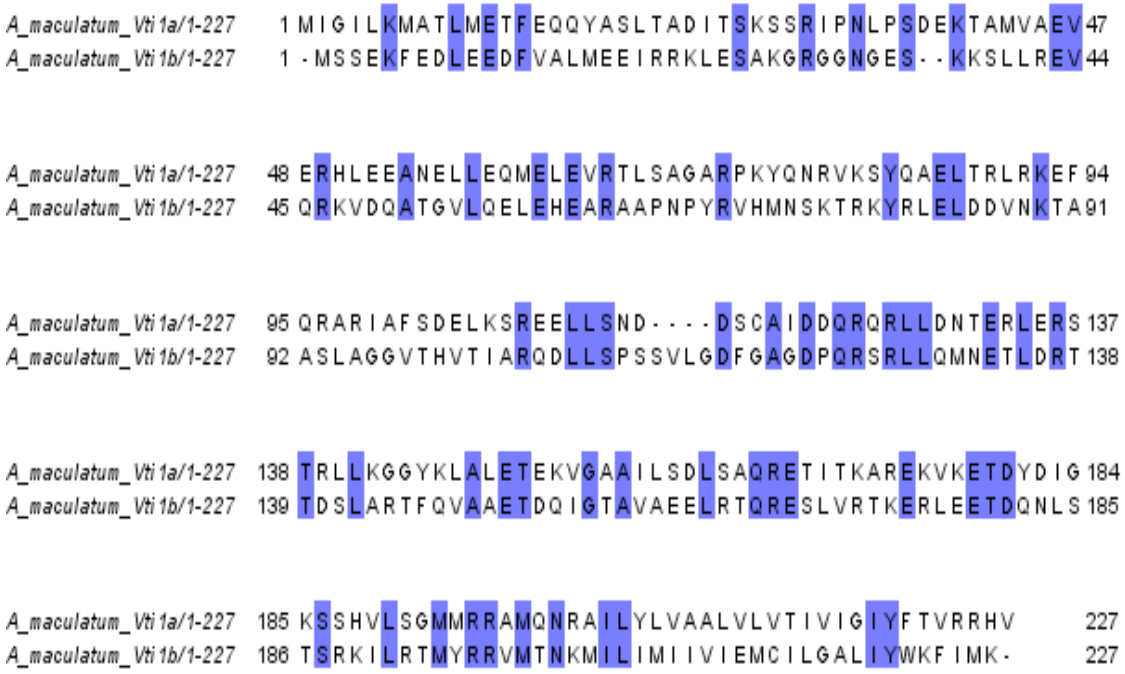

Figure 3. Amvti1A/Amvti1B Sequence Alignment. The *A. maculatum* Vti1a and Vti1b amino acid sequences were aligned with each other to illustrate the low homology between the two sequences. They contain 28% sequence identity (57% similarity).

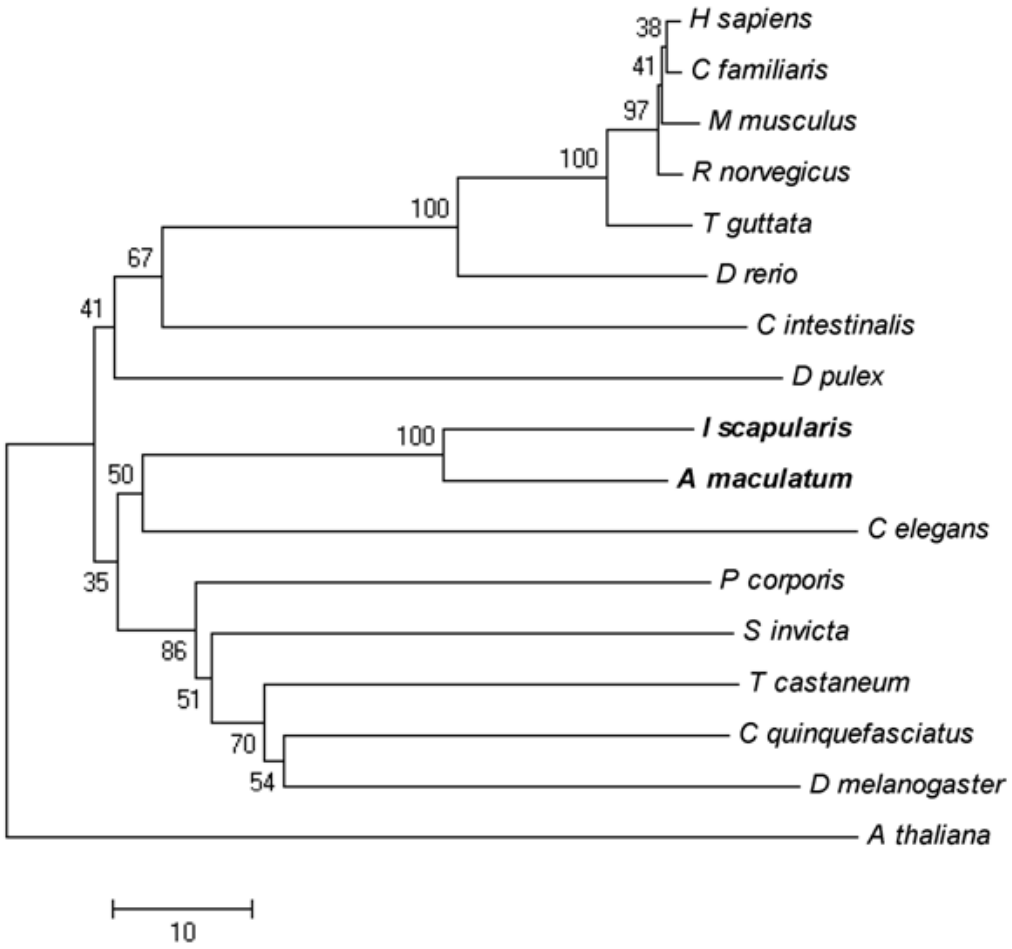

Figure 4 . Evolutionary relationships of taxa in consideration of Vti1A

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 528.86523438 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method [3] and are in the units of the number of amino acid differences per sequence. The analysis involved 17 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 160 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [4].

1. Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

2. Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

3. Nei M. and Kumar S. (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York.

4. Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution (In Press).

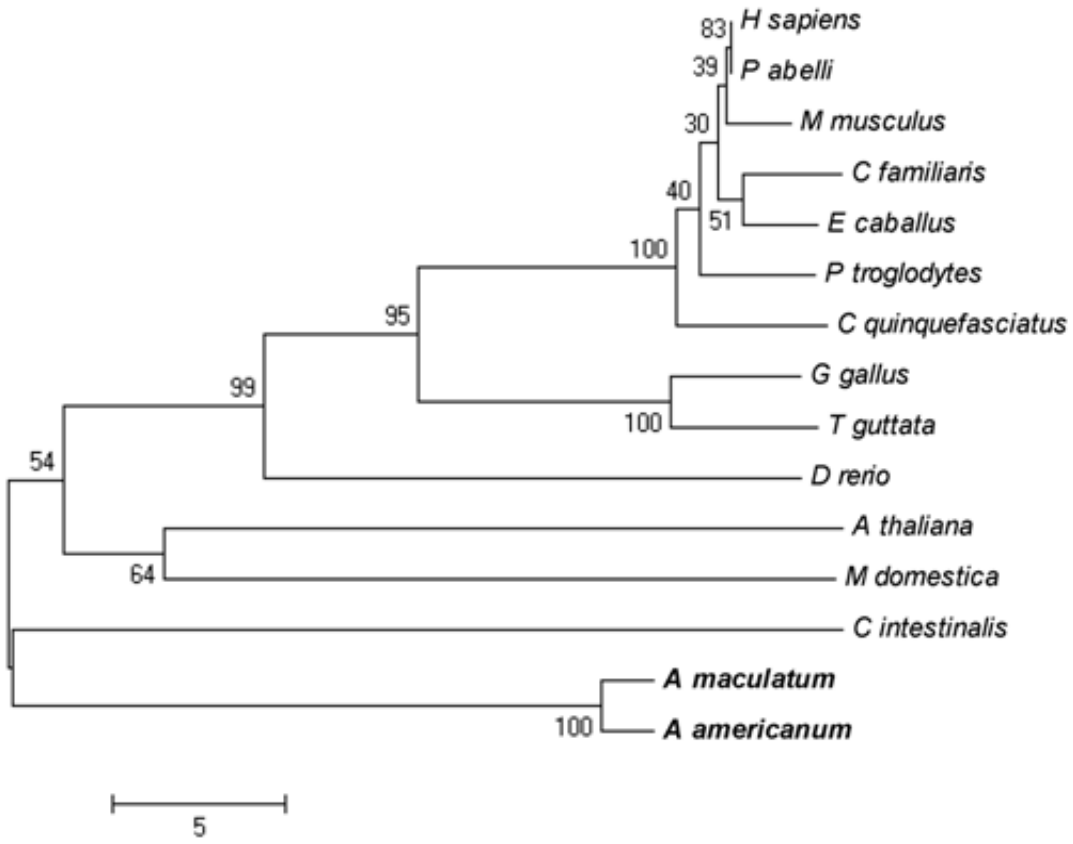

Figure 5. Evolutionary relationships of taxa in consideration of Vti1B

The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 152.98828125 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method [3] and are in the units of the number of amino acid differences per sequence. The analysis involved 15 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 57 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [4].

1. Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

2. Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

3. Nei M. and Kumar S. (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York.

4. Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution (In Press).

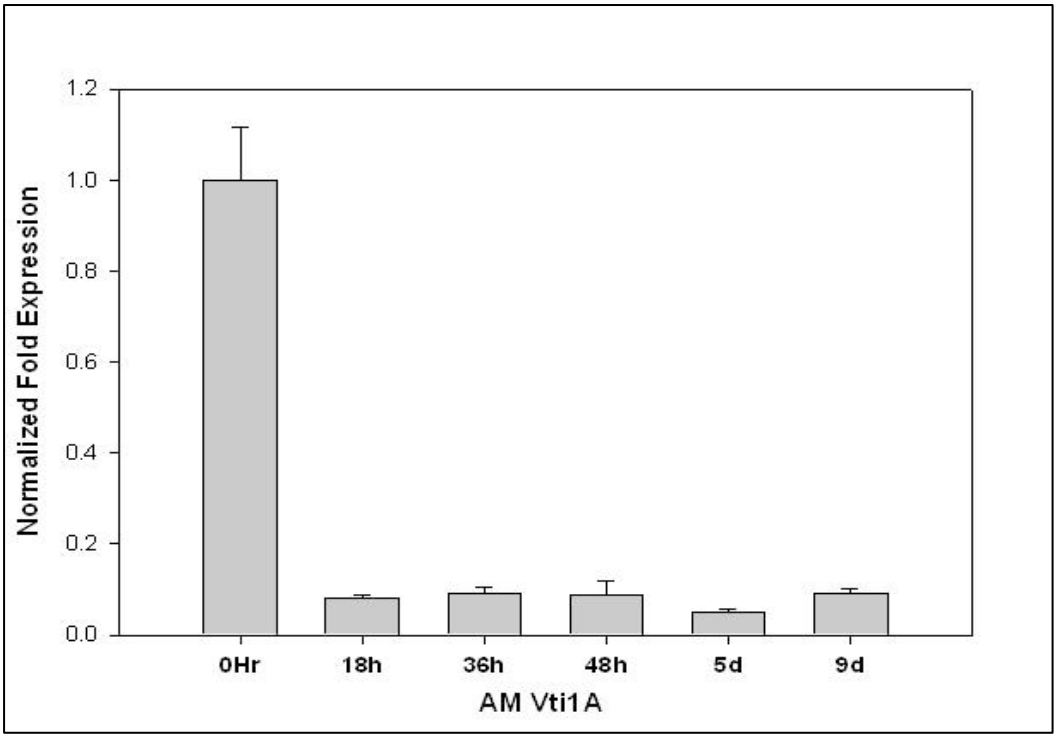

Figure 6. Transcriptional gene regulation of *Amblyomma maculatum* VTI1A in the salivary glands during the onset of tick blood feeding. The graph shows the difference in accumulation of transcripts for the aaVTI1B in the salivary glands as feeding continues from the unfed ticks. The expression was normalized using actin as house-keeping gene.

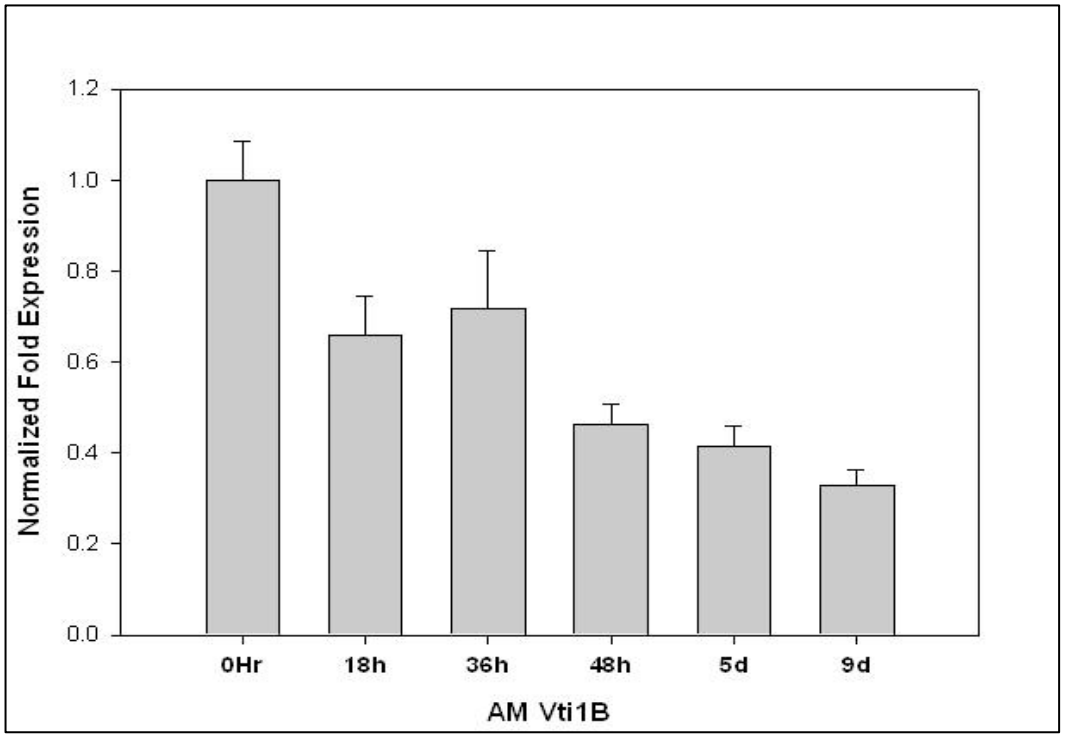

Figure 7: Transcriptional gene regulation of *Amblyomma maculatum* VTI1B in the salivary glands during the onset of tick blood feeding. The graph shows the difference in accumulation of transcripts for the aaVTI1B in the salivary glands as feeding continues from the unfed ticks. The expression was normalized using actin as house-keeping gene.

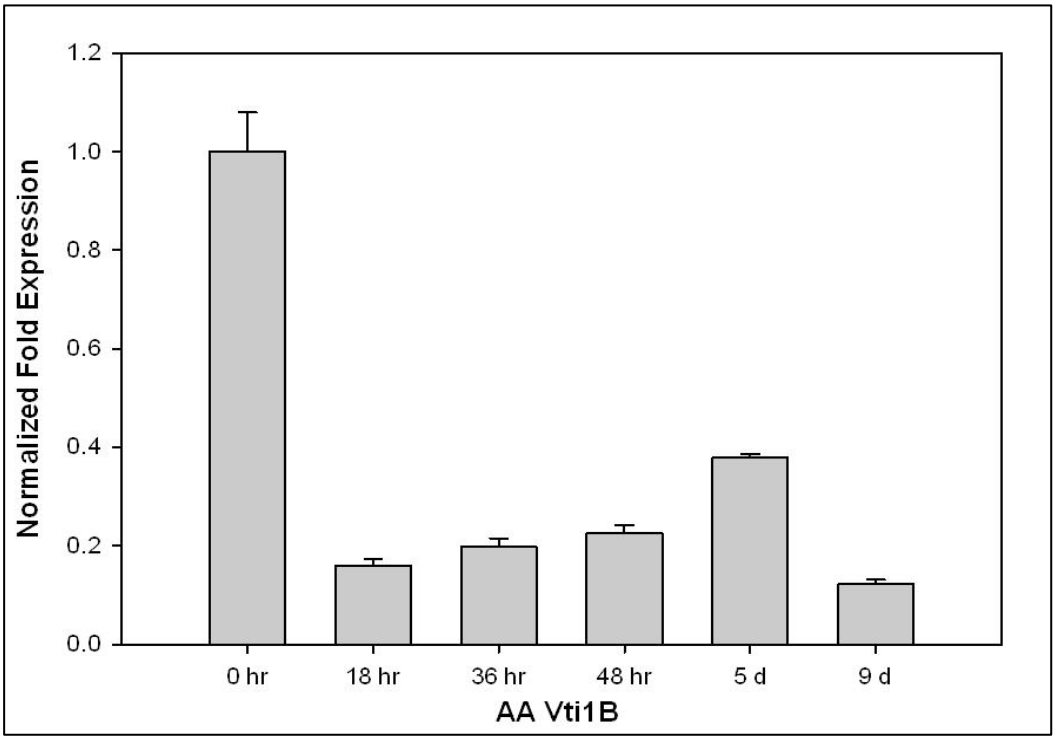

Figure 8: Transcriptional gene regulation of *Amblyomma americanum* VTI1B in the salivary glands during the onset of tick blood feeding. The graph shows the difference in accumulation of transcripts for the aaVTI1B in the salivary glands as feeding continues from the unfed ticks. The expression was normalized using calreticulin as house-keeping gene.

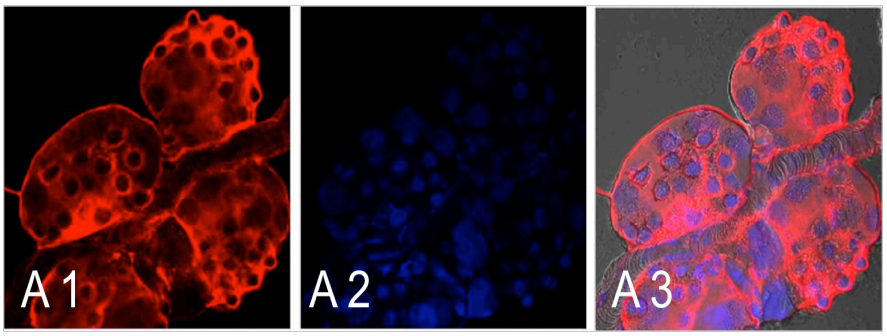

Figure 9: Immunolocalization of tick VTI1B in unfed salivary glands of *Amblyomma maculatum*. A1) VTI1B antibodies fluoresce red, A2) DAPI stained nuclei appear blue, A3) overlay of red and blue stain (20X magnification)

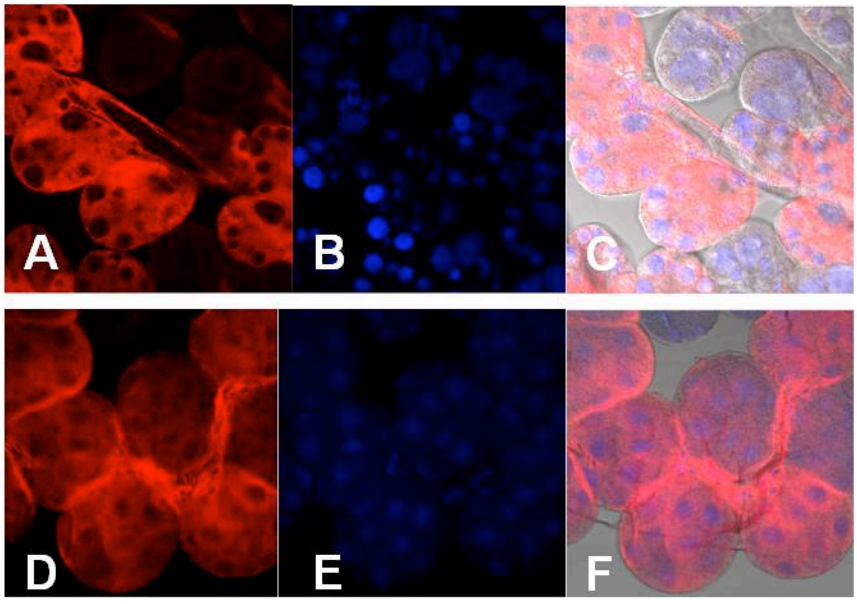

Figure 10: Immunolocalization of tick VTI1B in unfed (A-C) and partially fed (D-F) salivary glands of *Amblyomma americanum*. A-D) VTI1B antibodies fluoresce red, B-E) DAPI stained nuclei appear blue, C-F) overlay of red and blue stain (20X magnification).

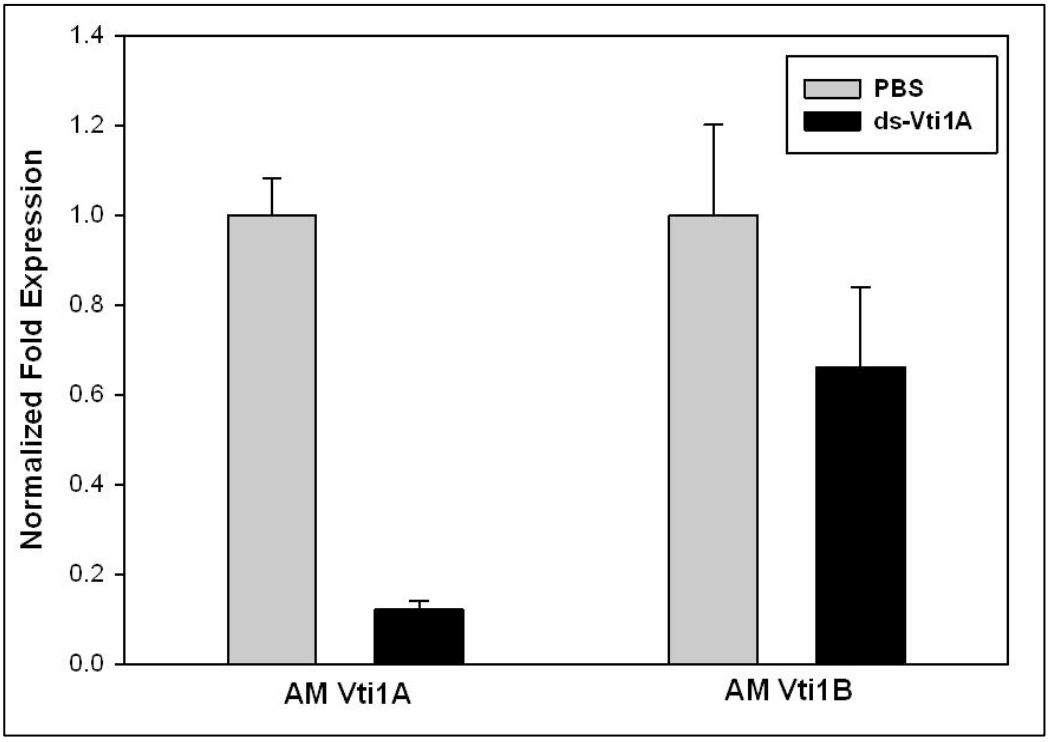

Figure 11: *In vivo* amVTI1A RNAi in the salivary glands of *A. maculatum*. Qualitative RT-PCR with total RNA prepared from irrelevant dsRNA injected control salivary glands or aaVTI1B RNAi salivary glands. The expression was normalized using actin as house-keeping gene. In ds-Vti1A treated ticks, Vti1A transcript levels were reduced by 88% and Vti1B transcript levels were reduced by 34%.

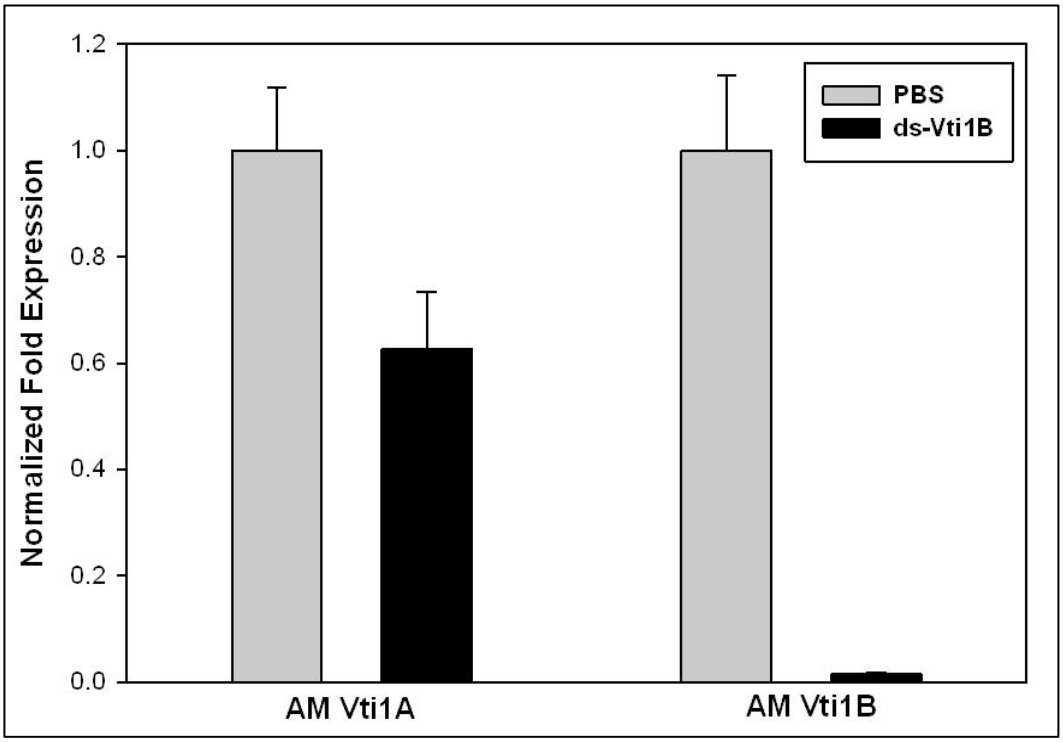

Figure 12: *In vivo* AM Vti1B RNAi in the salivary glands of *A. maculatum*. Qualitative RT-PCR with total RNA prepared from irrelevant dsRNA injected control salivary glands or aaVTI1B RNAi salivary glands. The expression was normalized using actin as house-keeping gene. In ds-Vti1B treated *A. maculatum* ticks, Vti1B transcript levels were reduced by 99% and Vti1A transcript levels were reduced by 37%.

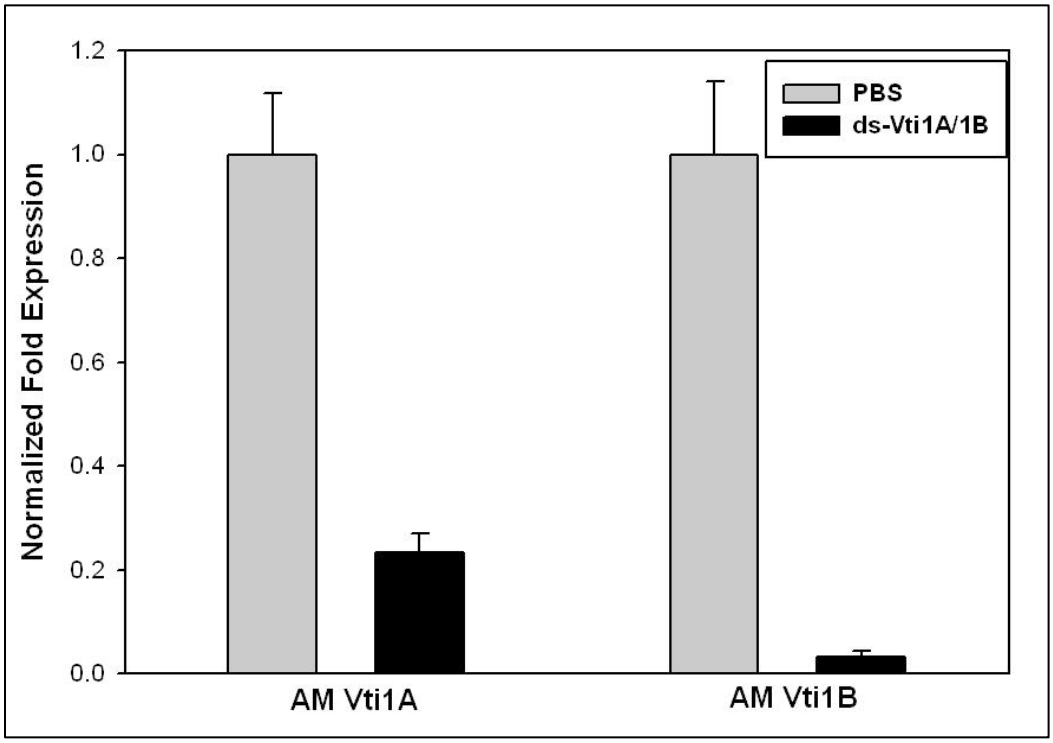

Figure 13: *In vivo* AM Vti1A/1B RNAi in the salivary glands of *A. maculatum*. Qualitative RT-PCR with total RNA prepared from irrelevant dsRNA injected control salivary glands or aaVTI1B RNAi salivary glands. The expression was normalized using actin as house-keeping gene. In DKO treated ticks, Vti1A transcript levels were reduced by only 77% whereas Vti1B transcript levels were reduced by 97%.

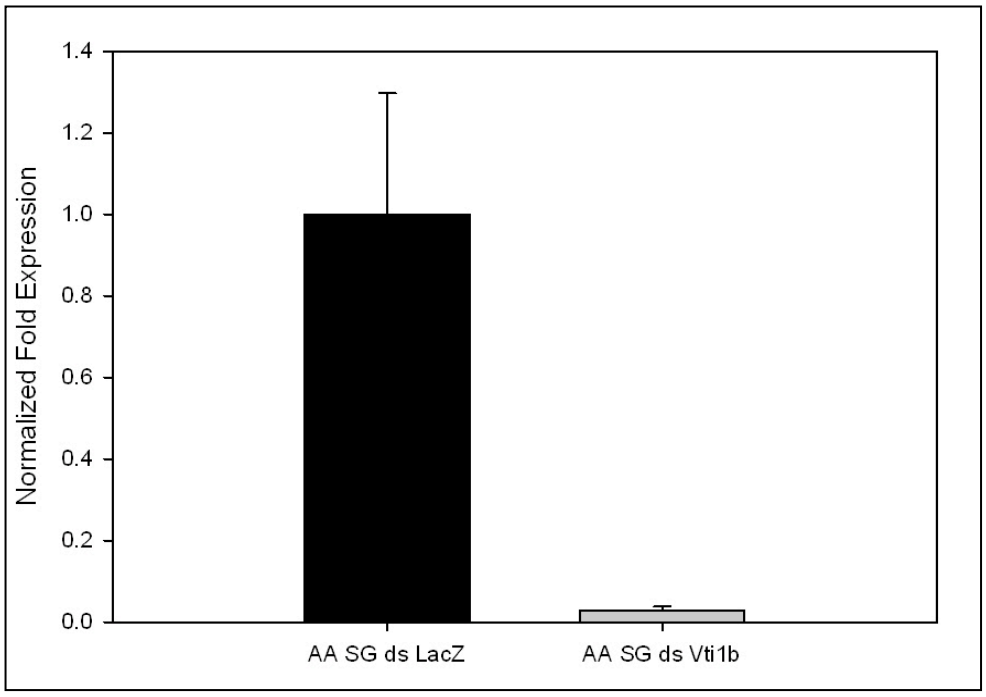

Figure 14: *In vivo* aaVTI1B RNAi in the salivary glands of *A. americanum*. Qualitative RT-PCR with total RNA prepared from irrelevant dsRNA injected control salivary glands or aaVTI1B RNAi salivary glands. The expression was normalized using calreticulin as house-keeping gene.

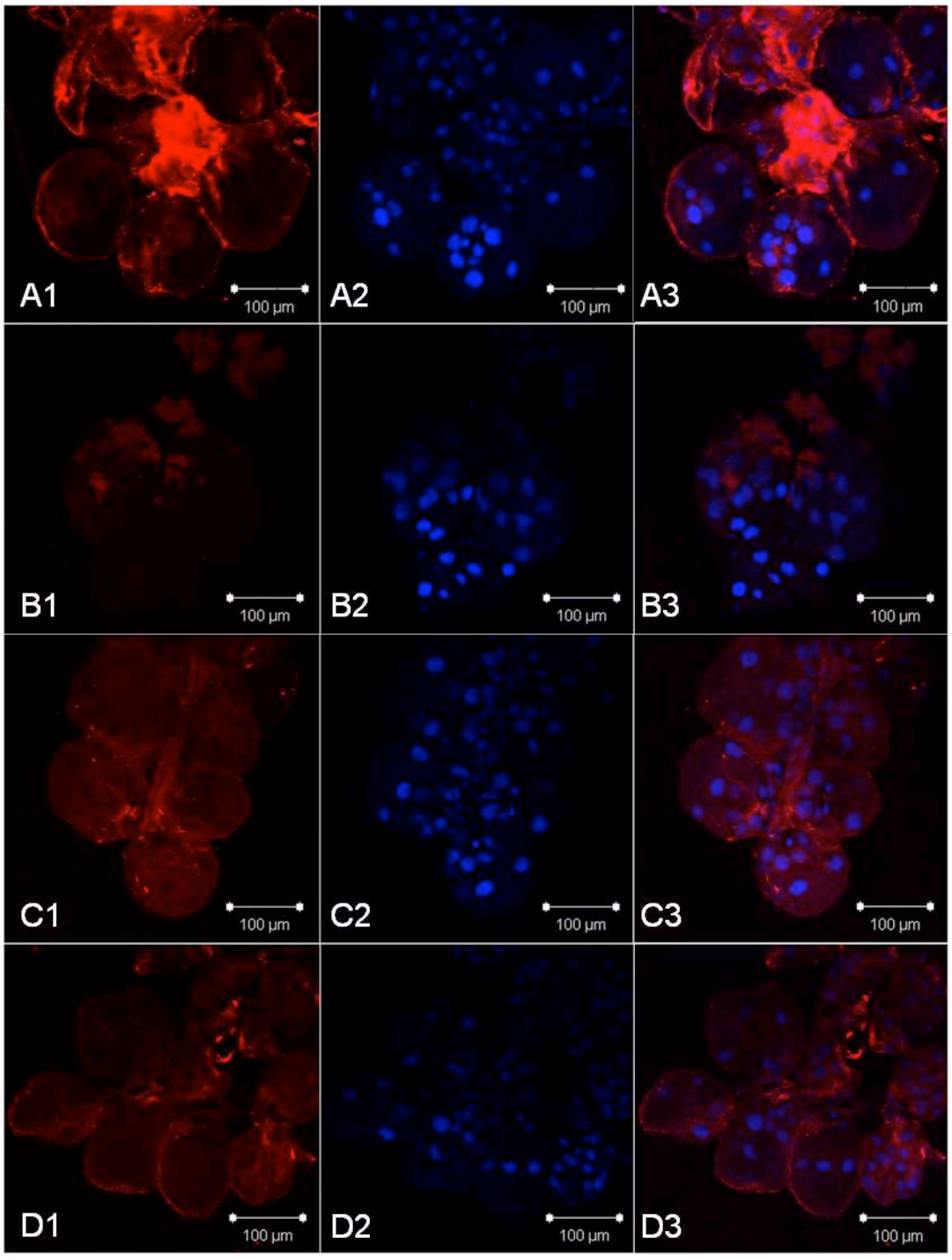

Figure 15: Immunolocalization of tick VTI1A in partially blood fed salivary glands (7d) of *Amblyomma maculatum* naïve ticks -injected with PBS (A1-A3), ticks injected with dsRNA-VTI1A (B1-B3), ticks injected with dsRNA-VTI1B (C1-C3), and ticks injected with dsRNA-VTI1A/1B (D1-D3). VTI1A antibodies stained red and DAPI stained nuclei blue.

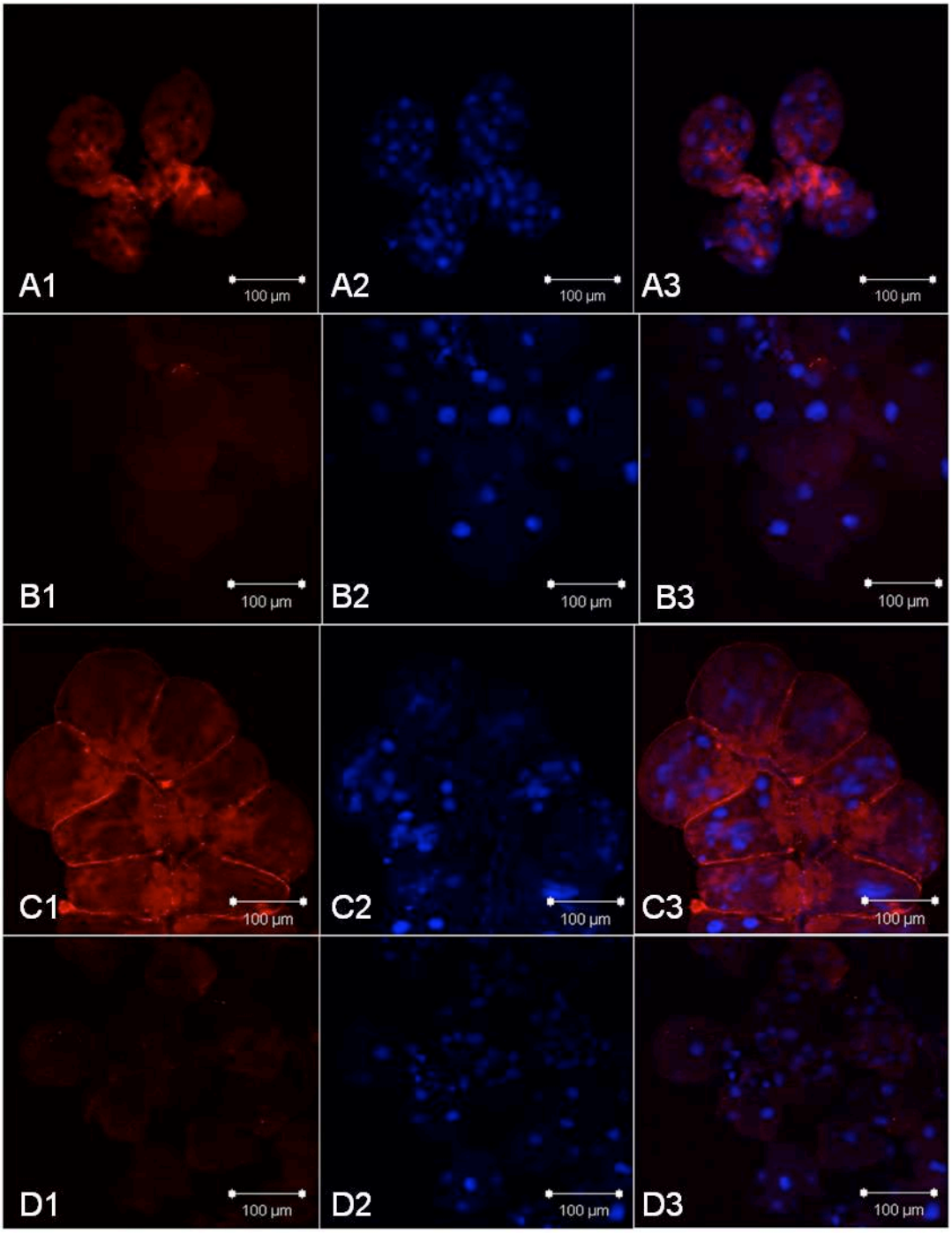

Figure 16: Immunolocalization of tick VTI1B in partially blood fed salivary glands (5d) of *Amblyomma maculatum* naïve ticks- injected with PBS (A1-A3), ticks injected with dsRNA-VTI1B (B1-B3), ticks injected with dsRNA-VTI1A (C1-C3), and ticks injected with dsRNA-VTI1A/1B (D1-D3). VTI1B antibodies stained red and DAPI stained nuclei blue.

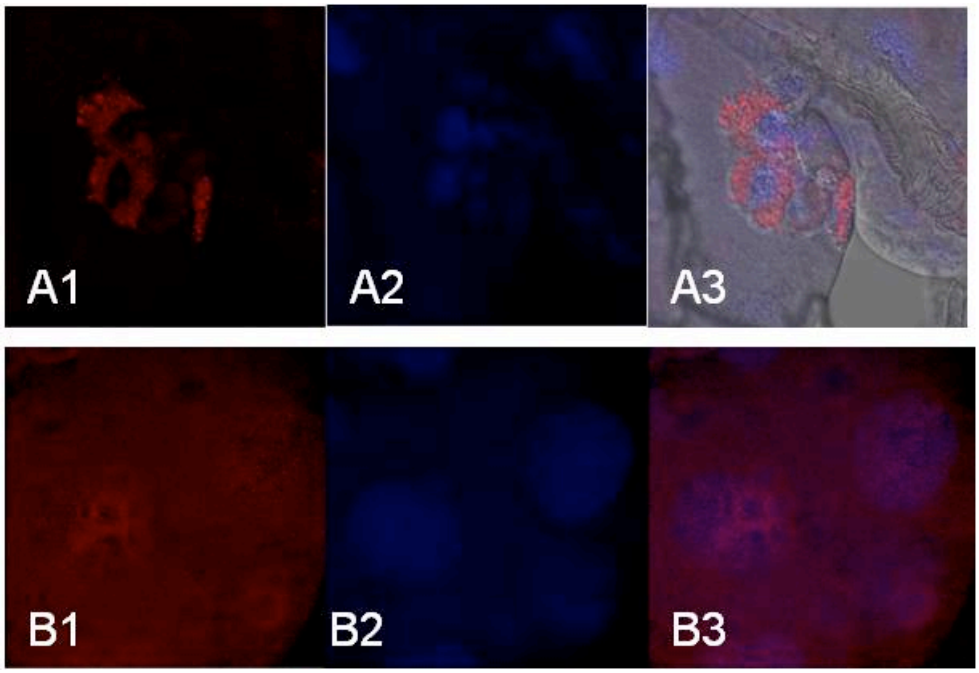

Figure 17: Immunolocalization of tick VTI1B in partially blood fed salivary glands of *Amblyomma americanum* naïve ticks- injected with irrelevant LacZ-dsRNA (A1-A3) and ticks injected with dsRNA-VTI1B (B1-B3). VTI1B antibodies stained red and DAPI stained nuclei blue.

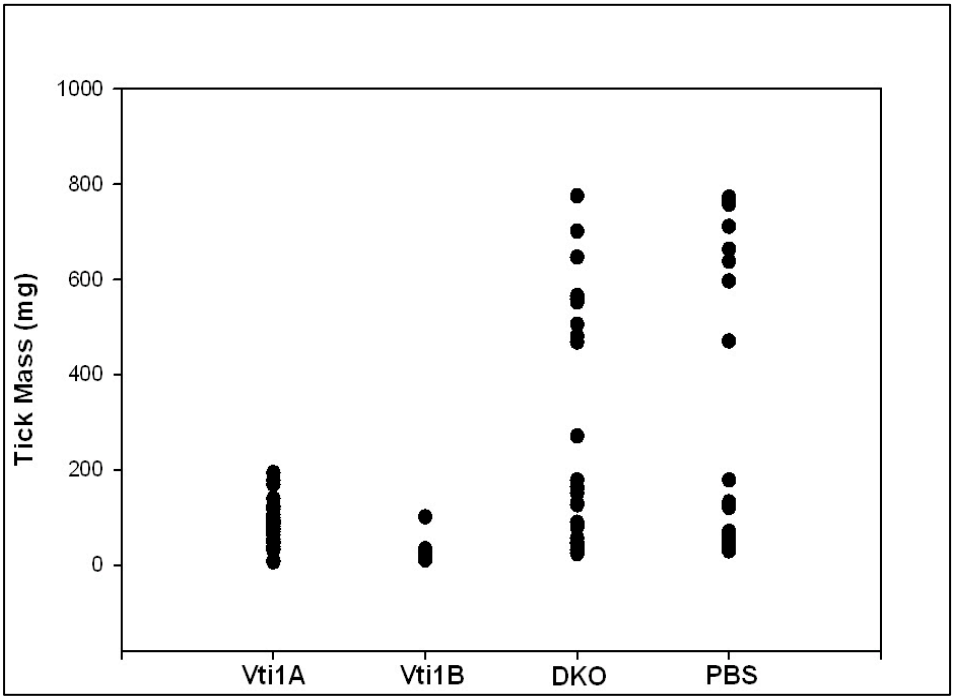

Figure 18: Individual tick masses (mg) of *Amblyomma maculatum* Vti1A, Vti1B and DKO RNAi. Comparison of average tick weight among ds-Vti1A, ds-Vti1B, ds-Vti1A/1B and ds-PBS treated ticks pulled from 6d-11d post attachment.

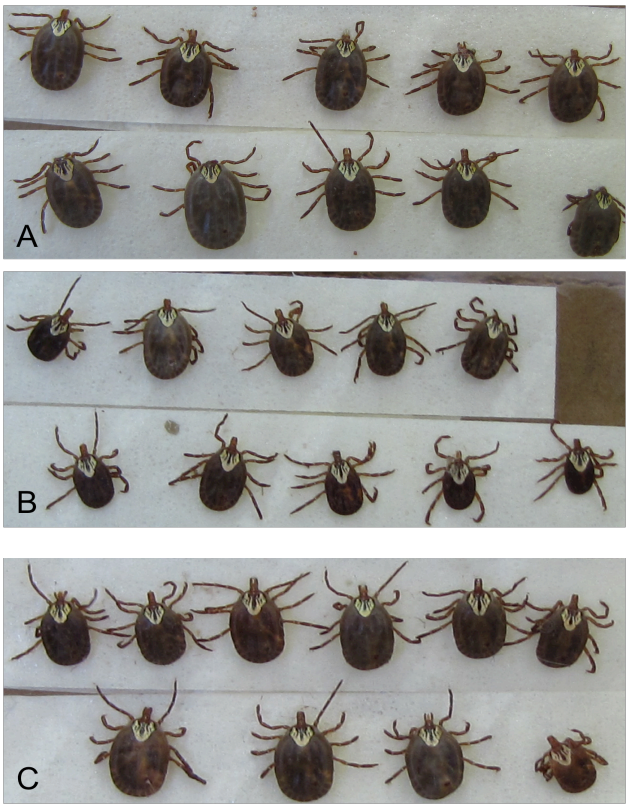

Figure 19: ***Amblyomma maculatum*** **Vti1B and DKO RNAi.** A) Partially fed dsRNA-PBS injected ticks after 6 days post-attachment B) dsRNA-AM Vti1B injected ticks 6 days post-attachment C) dsRNA-AM Vti1A/1B injected ticks 6 days post-attachment

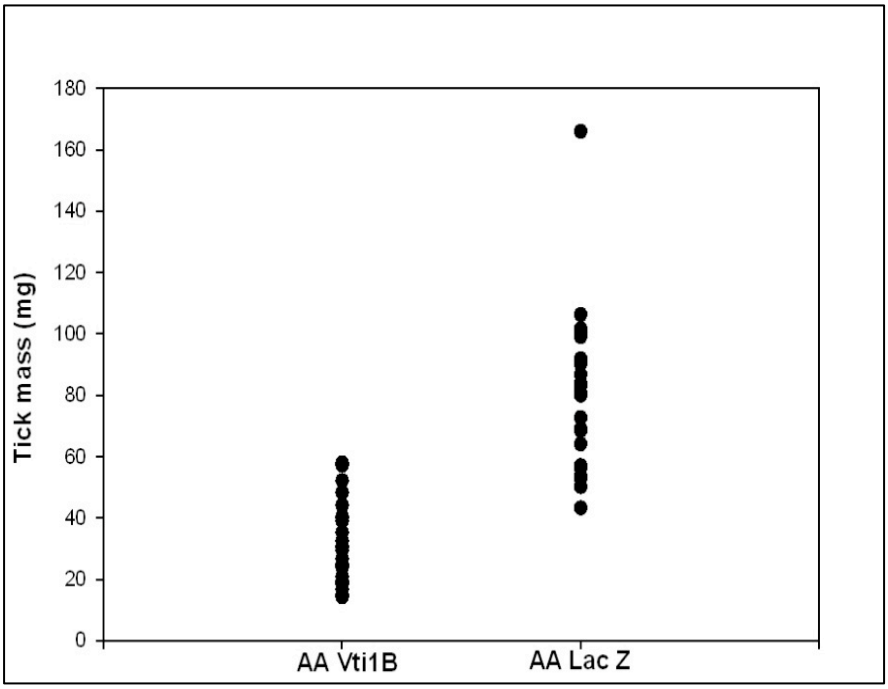

Figure 20: Individual tick masses (mg) of *Amblyomma americanum* Vti1B RNAi. Comparison of average tick weight between ds-Vti1B and ds-Lac Z treated ticks pulled from 6d-11d post attachment.

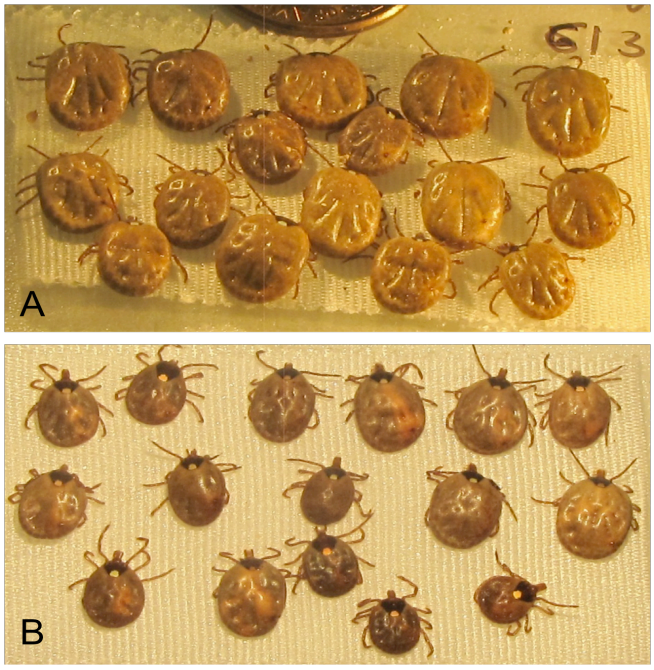

Figure 21: ***Amblyomma americanum*** **Vti1B RNAi.** A) Partially fed dsRNA-LacZ injected ticks after 5 days post-attachment B) dsRNA-AAVti1B injected ticks 5 days post-attachment

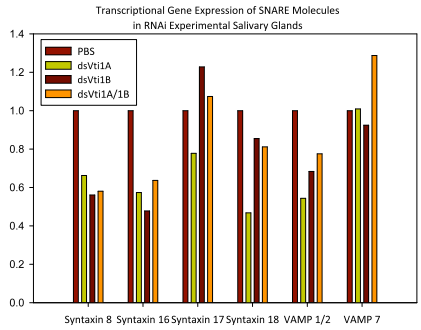

Figure 20: Transcriptional gene expression of various SNAREs in AM salivary gland dsRNA-Vti1A, Vti1B, and Vti1A/1B.

Gene	Qualitative/ Quantitative	Forward primer (5'-3')	Reverse primer(5'-3')
AM-Vti1A	Qualitative	ATGATTGGCATTCTGAAAATGGCG	TCACACATGCCTTCGAACTGTAAAA
AM-Vti1B	Qualitative	ATGTCCTCGGAAAAGTTTGAGGA	TCACTTCATGATAAACTTCCAGTAGATAA
AM-Vti1A	Quantitative	TGGAGCTTGAAGTCCGTACGTTGT	TCCTTAATCGTGTGAGTTCCGCCT
AM-Vti1B	Quantitative	AACTTTCCAAGTCGCCGCTGAAAC	TGTTGGTCATGACGCGCCTATACA
AM-Stx-8	Quantitative	TGCTAAACAAAGGGCGGGAAATGG	ACCACCTTCACTTACAGCCACACT
AM-Stx-16	Quantitative	TTGCAAGAGGCTTCAGTCCAGAGA	TGTTGATCTCCTCGTTCCCGCACTT
AM-Stx-17	Quantitative	TGGGCGCTGGATTCTTAGGTTACA	AACTGAGTGACTCGTCTGGTGGTGCTT
AM-Stx-18	Quantitative	AAGGCACCTCATGAGACAGAAGGA	ATCACCACATTCTCATCCCAGCCT
AM-VAMP1/2	Quantitative	AGGGTCAATACTGTGAACAGGCCA	ACTGATCCTCTTTGGAGCACGTCA
AM-VAMP 7	Quantitative	ACTTCTCGGAGGTCACCGAACAAA	ACTCACTTGGAACCTGCCCTGAAT
AM-Actin	Quantitative	TGGCTCCTTCCACCATGAAGATCA	TAGAAGCACTTGCGGTGCACAATG
AM-Tubulin	Quantitative	AACGCAGCTATTGCAGCCATCAAG	GTGGTGTTCGACAACATGCACACA
AA-Vti1B	Quantitative	TCGAAGAGGACTTTGCGGCACTAA	CTTTGCGCTGCACTTCACGAAGAA
AA-Calurectin	Quantitative	CGTCAAGCACGAGCAGAACATTGA	TGAGGTGGTTCTTGCCCTTGTAGT

Table 1. Primers used for Qualitative and Quantitative Analyisis.

References

Abeliovich, H., Darsow, T., and Emr, S.D. (1999). Cytoplasm to vacuole trafficking of aminopeptidase I requires a t-SNARE-Sec1p complex composed of Tlg2p and Vps45p. *EMBO J. 18***,** 6005–6016.

Advani,R.J., Bae, H-R., Bock, J.B., Chao, D.S., Doung, Y-C, Prekeris, R., Yoo, J-S, & Scheller, R.H. (1998). Seven Novel Mammalian SNARE Proteins Localize to Distinct Membrane Compartments. *Journal of Biological Chemistry, 273,* 10317-10324.

Antonin, W., Riedel, D. & Fischer von Mollard, G. (2000). The SNARE *Vti1a-β* Is Localized to Small Synaptic Vesicles and Participates in a Novel SNARE Complex. *The Journal of Neuroscience, 20(15)*, 5724-5732.

Antonin, W., Holroyd, C., Fasshauer, D., Pabst, S., Fischer von Mollard, G. & Jahn, R. (2000). A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. *The EMBO Journal, 19,* 6453-6464.

Armstrong PM, Rich SM, Smith RD, Hart DL, Spielman A, Telford SR III. (1996)A new *Borrelia* infecting lone star ticks. Lancet; 347:67-8.

Atlashkin, V., Kreykenbohm, V., Eskelinen, E-L., Wenzel, D., Fayyazi, A., & Fischer von Mollard, G.(2003). Deletion of the SNARE *vti 1b* in Mice Results in the Loss of a Single SNARE Partner, Syntaxin 8. *Molecular and Cellular Biology, 23*(15), 5198-5207.

Barbour AG, Maupin GO, Teltow GJ, Carter CJ, Piesman J. (1996). Identification of an uncultivatable *Borrelia* species in the hard tick*Amblyomma americanum*: possible agent of a Lyme disease–likeillness. J Infect Dis;173:403-9.

Bennet, M.K. & Scheller, R.H. 1993. The molecular machinery for secretion is conserved from yeast to neurons. *Proceedings of the National Academy of Sciences USA, 90*, 2259-2563.

Bethani,I., Werner, A., Kadian, C., Geumann,U., Jahn, R., & Rizzoli, S.O. 2009. Endosomal Fusion upon SNARE Knockdown is Maintained by Residual SNARE Activity and Enhanced Docking. *Traffic, 10(10),* 1543-1559.

Bishopp FC, Hixson H. (1936). Biology and economic importance of the Gulf Coast tick. J. Econ. Entomol. 29, 1068-1076.

Bishopp FC & Trembley HL. (1945). Distribution and hosts of certain North American ticks. *J. Parasitol. 31*, 1-54

Bose, A., Guilherme, A., Huang, S., Hubbard, A.C., Lane, C.R., Soriano, N.A. & Czech, M.P. (2005). The v-SNARE *Vti1a* Regulates Insulin-stimulated Glucose Transport and Acrp30 Secretion in 3T3-L1 Adipocytes. *The Journal of Biological Chemistry, 280(44)*, 36946-36951.

Bowman,A.S., Nuttall,P.A. and Chappell, L.H. (2004). Ticks: Biology, Disease and Control. *Parasitology, 129*, S1-S1.

Bowman, A.S. & Sauer, J.R. (2004). Tick salivary glands: function, physiology, and future. *Parasitology, 129,*S67-S81.

Burridge MJ.(1997). Heartwater: an increasing serious threat to the livestock and deer populations of the United States. In Proceedings of the 10th Annual Meeting of the U. S. Animal Health Association, Spectrum, Richmond, VA.

Campbell GL, Paul WS, Schriefer ME, Craven RB, Robbins KE. (1995). Epidemiologic and diagnostic studies of patients with suspected early Lyme disease, Missouri, 1990–1993. *J Infect Dis 17,* 470-80.

Centers for Disease Control and Prevention. *Recommendations for the use of Lyme disease vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Morb Mortal Wkly Rep48 RR-7:1-17. 21–25 (erratum: MMWR Morb Mortal Wkly Rep 1999; 48:833).*

Childs, J. E., & Paddock C. D. (2003). The ascendancy of amblyomma americanum as avector of pathogens affecting humans in the United States. *Annual Review of Entomology. 48,* 307-337.

Cooley RA &Kohls GM, (1944). The genus *Amblyomma* (Ixodidae) in the United States. *J. Parasitol. 30*, 77-111.

Cupp EW. (1991). Biology of Ticks, Veterinary Clinics of North America, Small Animal Practice, Hoskins, J.D.(Ed.), 21:1

Dressel, R., Elsner, L., Novota, P., Kanwar, N., Fischer von Mollard, G. (2010). The Exocytosis of Lytic Granules Is Impaired in Vti1b- or Vamp8-Deficient CTL Leading to a Reduced Cytotoxic Activity following Antigen-Specific Activation. *The Journal of Immunology, 2010 (185)*, 1005-1014.

Drummond RO & Whetstone TM. (1970). Oviposition of the Gulf Coast tick. *J. Econ. Entomol. 63*, 1547-1551.

Espinoza-Gomez F, Newton-Sanchez O, Flores-Casares G, De la Cruz-Ruiz M, Melnikov V, Austria-Tejeda J, Rojas-Larios F. 2011. Tick Paralysis caused by *Amblyomma maculatum* on the Mexican Pacific Coast. Vector Borne Zoonotic Dis. , [Epub

ahead of print].

Estrada-Pena A., Venzal JM, Mangold AJ, Cafrune MM, Guglielmone AA. (2005). The *Amblyomma maculatum* Koch, 1844 (Acari: Ixodidae: *Amblyomminae*) tick group: diagnostic characters, description of the larva of A. parvitarsum Neumann, 1901, 16S rRNA sequences, distribution and hosts. *Syst Parasitol. 60*, 99-112.

Fasshauer, D., Sutton, R.B., Brunger, A.T. & Jahn, R. (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc. Natl Acad. Sci. USA, 95*, 15781-15786.

Fischer von Mollard, G., and Stevens, T.H. (1999). The *Saccharomyces cerevisiae* v-SNARE Vti1p is required for multiple membrane transport pathways to the vacuole. *Mol. Biol. Cell 10***,** 1719–1732.

Francischetti, I.M.B., Sá-Nunes, A., Mans, B.M., Santos, I.M., & Ribeiro, M.C. (2009). The role of saliva in tick feeding. *Front Biosci. 14*, 2051-2088.

Goddard, J. & Norment, B. R., (1983). Notes on the geographical distribution of the Gulf Coast tick, *Amblyomma maculatum* (Koch) [Acari: Ixodidae]. *Entomol. News. 94,* 103-104.

Goddard, J.,& Varela-Stokes, A.S. (2008). Role of the lone star tick, *Amblyomma americanum* (L.), in human and animal diseases. *Vet. Parasitol.* doi:10.1016/ j.vetpar. 10.089

Greiner EC, Humphrey PP, Belden RC, Frankenberger WB, Austin DH, Gibbs EPJ. (1984). Ixodid ticks on feral swine in Florida. *J. Wild Dis. 20*, 114-119.

Hair JA, Bowman JL. Behavioral ecology of Amblyomma americanum (L.). In: Sauer RJ, Hair HA, editors. *Morphology, physiology, and behavioral biology of ticks*. p. 407.

Hair J.A., Howell D.E. (1970). Lone star ticks. Their biology and control in Ozark recreation areas. Rep. Bull. B-679, Oklahoma State Univ., Agric. Exp. Sta., Okia

Hay, J.C. & Scheller, R.H. (1997). SNAREs and NSF in targeted membrane fusion. *Current Opinion in Cell Biology, 9*, 505-512.

Hong W (2005) SNAREs and traffic. *Biochim Biophys Acta, 1744*, 120–144

Hooker WA, Bishopp FC, Wood HP. (1912). The life history and bionomics of some North American ticks. US Dep. Agr. Bur. Entomol. Bull. 106.

Hovius, J., Levi, M., & Fikrig, E. (2008). Salivating for Knowledge: Potential Pharmalogical Agents in Tick Saliva. *PLoS Medicine*, 5 (*2*), 202-208.

Jahn, R. & Scheller, R.H. (2006). SNAREs-engines for membrane fusion. *Nature Reviews Molecular Cell Biology, 7,* 631-643.

Karim, S. & Adamson, S. (2012). RNA Interference in Ticks: A Functional Fenomics Tool for the Study of Physiology. *Advances in Insect Physiology, 42*, 119-146.

Karim, S., Essenber, R.C., Dillwith, J.W., Tucker, J.S., Bowman, A.S., Sauer, J.R. (2002). Identification of SNARE and cell trafficking regulatory proteins in the salivary glands of the lone star tick, *Amblyomma americanum*. *Insect Biochemistry and Molecular Biology, 32 (12),* 1711-1721.

Karim S , Singh P , Ribeiro JMC (2011) A Deep Insight into the Sialotranscriptome of the Gulf Coast Tick, *Amblyomma maculatum*. PLoS ONE 6(12): e28525. doi:10.1371/journal.pone.0028525

Keirans JE & Lacombe EH. (1998). First records of Amblyomma americanum, Ixodes (Ixodes) dentatus, and Ixodes (Ceratixodes) uriae (Acari Ixodidae) from Maine. *J. Parasitol. 84,* 629-31.

Kirkland KB, Klimko TB, Meriwether, R.A., Schriefer, M., Levin, M., Levine, J., Mac Kenzie, W.R., & Dennis, D.T. (1997). Erythema migrans–like rash illness at a camp in North Carolina:a new tick-borne disease?. *Arch Intern Med 15,* 2635-2641.

Kreykenbohm,V., Wenzel, D. Antonin, W. Atlachkine, V., & Fischer von Mollard, G. (2002). The SNARES vti1a and vti1b have distinct localization and SNARE complex partners. *European Journal of Cell Biology, 81*, 273-280.

Kunwar, A.J., Rickmann, M., Backofen, B., Browski, S.M., Rosenbusch, J., Schoing, S., … Fischer von Mollard,G. (2010). Lack of the endosomal SNAREs vti1a and vti1b led to significant impairments in neuronal development. *PNAS, Early Edition*, 1-6.

Lees, K. & Bowman, A.S. (2007). Tick neurobiology: recent advances and the post-genomic era. *Invert. Neuroscience, 7,* 183-198.

Mahan, S. M., T. F. Peter, B. H. Simbi, K. Kocan, E. Camus, et al., (2000). Comparison of efficacy of American and African *Amblyomma* ticks as vectors of heartwater (*Cowdria ruminantium*) infection by molecular analysis and transmission trials. *J. Parasitol. 86*, 44-49.

McMullen, H.L., Sauer, J.R., & Burton, R.L. (1976). Possible role in uptake of water vapour by ixodid tick salivary glands. *Journal of Insect Physiology*, *22 (9)*, 1281-1285.

Paddock CD, Summer JW, Comer JA, Zaki SR, Goldsmith CS, Godard J, et al., (2004). *Rickettsia parkeri*: a newly recognized cause of spotted fever rickettsiosis in the United States. *Clin. Infect. Dis. 38*, 805-811.

Paddock CD, Fournier PE, Sumner JW, Goddard J, Elshenawy Y, Mecafe MG, Loftis AD, Varela-Stokes A. (2010). Isolation of *Rickettsia parkeri* and identification of a novel spotted fever group Rickettsia sp. from Gulf Coast ticks (*Amblyomma maculatum*) in the United States. *Appl. Environ. Microbiol. 76*, 2689-2696.

Paffenbarger RS Jr. (1951). Tick paralysis; implicating *Amblyomma maculatum*. *New Orleans Med Surg J 103*, 329-332.

Parker RR, Kohls GM, Cox GW, Davis GE. (1939). Observations on an infectious agent from *Amblyomma maculatum*. *Public Health Rep. 54*, 1482-1484.

Patrick, C.D. & Hair, J.A. (1975). Laboratory rearing procedures and equipment for multi-host ticks (Acari: Ixodidae). *Journal of Medical Entomology, 12*, 389-390.

Ribeiro, J.M.C & Francischetti, I.M.B. (2003). Role of arthropod saliva in blood feeding: Sialome and post-sialome perspectives. *Annual Review of Entomology, 48,* 73-88.

Robinson LE. 1926. The genus Amblyomma , pp. 40-44, 285-286. In G. H. F. Nuttall, C. Warburton and L. E. Robinson (eds.), Ticks: A monograph of the Ixodidae, vol. II. Cambridge University Press, London, United Kingdom.

Rudolph, D. & Knulle, W. (1974). Site and mechanism of water vapour uptake from the atmosphere in ixodid ticks. *Nature, 249 (5452)*, 84-85.

Sauer, J.R., Essenberg, R.C., Bowman, A.S. (2000). Salivary glands in ixodid ticks: control and mechanism of secretion. *J. Insect Physiol. 46,* 1069-1078.

Sauer, J.R., McSwain, J.L., Bowman, A.S. (1995). Tick salivary gland physiology. *Annu. Rev. Entomol. 40,* 245-267.

Sauer, J. R. & Hair, J. A. (1986) *Morphology*, *Physiology*, *and Behavioral Biology of Ticks* (Halsted Press, New York).

Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., Rothman, J.E. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature 362,* 318-324.

Sonenshine D. 1993. Biology of ticks. Volume 2. Oxford University Press, New York.

Strickland RK, Gerrish RR, . Hourrigan JL, Schubert GO. (1976). Ticks of veterinary importance. US Dept. Agric. Handb.p. 485.

Sumner, John W., Lance Durden, Jerome Goddard, Ellen Stomdahl, Kerry Clark, Will Reeves and Christopher Paddock. Gulf Coast Ticks (*Amblyomma maculatum)* and *Rickettsia parkeri*, United States. *Emerging Infectious Diseases online.*13.5 (2007) 751-75e.Web. 23 Feb 2011.

Surpin, M., Zheng, H., Morita, M.T., Saito, C., Avila, E., Blakeslee, J.J., …Raikhel, N. (2003). The VTI Family of SNARE Proteins is Necessary for Plant Viability and Mediates Different Protein Transport Pathways. *The Plant Cell, 15*, 2885-2899.

Tatchell, R.J. (1967). Salivary secretion in the cattle tick as a means of water elimination *Nature (London) 213*, 940-941.

Teel PD, Ketchum HR, Mock DE, Wright RE, and Strey OF. 2010. The Gulf Coast tick: a review of the life history, ecology, distribution, and emergence as an arthropod of medical and veterinary importance. J. Med. Entomol. 47, 707-722.

USDA APHIS (United States Department of Agriculture Animal and Plant Health

Inspection Service)., 2008. Assessment of change in risk for release of *Ehrlichia ruminantium* (Heartwater) into the continental United States following discontinuation of the Tropical Bont Tick (Amblyomma variegatum) eradication program on St. Croix, U.S. Virgin Islands. Centers for Epidemiology and Animal Health. Ft. Collins CO.

Walker JB & Olwage A. (1987). The tick vector vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus *Amblyomma*) and their distribution. *Onderstepoort J. Vet. Res. 54*, 353-379.

Wang G, van Dam AP, Schwartz I, Dankert J.(1999). Molecular typing of Borrelia burgdorferi sensu lato: taxonomic, epidemiological, and clinical implications. *Clin Microbiol Rev 12,* 633-53.

Williams HR, Hair JA, McNew RW. (1978). Effect of Gulf Coast ticks on blood composition and weights of pastured Hereford steers, *J. Parasitol. 64*, 336-342.

Wright CL, Nadolny RM, Jiang J, Richards AL, Sonenshine DE, Gaff HD, Hynes WL. (2011). *Rickettsia parkeri* in gulf coast ticks, southeastern Virginia, USA. Emerg. *Infect Dis 17*, 896-898.